University of Alberta

The source and fate of protons in postischemic hearts

By

Que Liu ©

A thesis submitted to the Faculty of Graduates Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Department of Pharmacology

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Abstract

Introduction: Intracellular acidosis is one of the major triggers for ischemia/ reperfusion injury. This is because the Na^+/H^+ exchanger (NHE1) is activated during reperfusion, resulting in intracellular Na^+ and Ca^{2+} overload. High rates of fatty acid oxidation during reperfusion may contribute to NHE1 activation by increasing proton (H^+) production due to an uncoupling of glycolysis with glucose oxidation. This is associated with poor recovery of cardiac function and efficiency. The present studies determined if increased H^+ production delays the recovery of intracellular pH (pH_i) during reperfusion and whether reducing H^+ production or inhibiting NHE1 can improve the recovery of cardiac function and efficiency

Methods: An isolated working heart model for measurement of pH_i by ^{31}P-NMR was developed. Hearts perfused with [5-^{3}H/U-^{14}C]glucose (5.5 mM) ± [1-^{14}C]palmitate (1.2 mM) were subjected to 30 min aerobic perfusion, 20 min of global no-flow ischemia and 40 or 50 min of reperfusion.

Results: In the presence of palmitate, glucose oxidation was inhibited, causing an increased H^+ production from uncoupled glucose metabolism. Treatment with T_3 stimulated glucose oxidation, reduced H^+ production and improved the recovery of cardiac function and efficiency. If hearts were perfused with glucose alone, or with dichloroacetate (to stimulate glucose oxidation), which significantly reduced H^+ production by improving the coupling of glucose metabolism, accelerated pH_i recovery and improved recovery of cardiac function and efficiency were seen during reperfusion. Inhibition of NHE1 by cariporide also

improved the recovery of cardiac function and efficiency, despite a slower recovery of pH_i. H_2O_2 did not decrease pH_i by improving the coupling of glucose metabolism.

Conclusion: An increased H^+ production generated from uncoupled glucose metabolism, due to the presence of a high level of fatty acid, significantly delays the recovery of pH_i during reperfusion and contributes to the poor recovery of cardiac function and efficiency. Either reducing H^+ production or inhibition of NHE1 improves the recovery of cardiac efficiency and contractile function. The recovery of pH_i *per se* is not critical for the recovery of cardiac mechanical function and efficiency. It is the clearance of H^+ via the NHE1 that contributes to ischemia/reperfusion injury.

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List of abbreviations

ACC:	acetyl CoA carboxylase
ADP:	adenosine diphosphate
AE:	Cl^--HCO_3^- exchanger
AMP:	adenosine monophosphate
AMPK:	5'-AMP-activated protein kinase
ATP:	adenosine monophosphate
CA:	carbonic anhydrase
CAR:	cariporide
CoA:	coenzyme-A
CPT I:	carnitine palmitoyltransferase I
CPT II:	carnitine palmitoyltransferase II
CVR:	coronary vascular resistance
DCA:	dichloroacetate
FID:	free induction decay
GAPDH:	glyceraldehyde 3-phosphate dehydrogenase
H^+:	proton(s)
LDH:	lactate dehydrogenase
LPL:	lipoprotein lipase
MCD:	Malonyl-CoA decarboxylase
MCT:	monocarboxylate carrier
MVO_2:	Myocardial O_2 consumption
NAD^+:	nicotinamide adenine nucleotide

NADH:	dihydronicotinamide adenine nucleotide
NCE:	Na^+/Ca^{2+} exchange
NHE:	Na^+/H^+ exchanger
NHE1:	housekeeping isoform of Na^+/H^+ exchanger
NMR:	nuclear magnetic resonance
PCr:	phosphocreatine
PDH:	pyruvate dehydrogenase
PDHa:	active PDH
PDHt:	total PDH
PFK:	phosphofructokinase
pH_i:	intracellular pH
Pi:	inorganic phosphate
T_3:	3,5,3'-triiodo-L-thyronine
TCA:	tricarboxylic acid
VLDL:	very low-density lipoprotein

Chapter 1

Introduction

A reduction in blood flow to the heart (termed cardiac ischemia) depresses cardiac contractility. These severe reductions in blood flow potentially lead to cell death. This is a serious condition, but it can usually be treated successfully by an early restoration of blood flow (reperfusion), either by by-pass surgery, thrombolytic drugs or angioplasty. However, reperfusion does not always result in a complete recovery of contractile function. It is well known that reperfusion is accompanied by detrimental manifestations known as "reperfusion injury" (see 1 for review). Reperfusion injury refers to an event associated with reperfusion that had not occurred during the preceding ischemic period and can be entirely attenuated by an intervention given during ischemia and reperfusion. It classically includes myocardial stunning, reperfusion arrhythmia and lethal reperfusion injury. This research is concerned with mechanisms that limit the beneficial actions of reperfusion, particularly the sources and the consequences of the excessive production of protons (H^+) during the critical early period of reperfusion. Specifically, both the production (source) and clearance (fate) of H^+ in isolated working hearts were pharmacologically altered under carefully controlled conditions of energy supply and demand. In this way, the effects of intracellular H^+ on cardiac mechanical function and efficiency during reperfusion were investigated. A greater knowledge of the role of H^+ in reperfusion will help us to formulate strategies to improve the treatment of patients with ischemic diseases.

1. Cardiac energy metabolism and the source of H^+

As with most living tissue, heart muscle utilizes energy in the form of adenosine triphosphate (ATP), at a rate of about 200 μmol • g dry wt^{-1} • min^{-1}. As the content of ATP is normally about 30 μmol • g dry wt^{-1} , ATP must be resynthesized as quickly as it is consumed[2-4]. Without continuous replenishment, intracellular stores of ATP would be exhausted in < 1 min. This concept finds its expression in the ATP cycle simply depicted in the following equations:

$$\text{ATP} \leftrightarrows \text{Adenosine diphosphate (ADP)} + \text{inorganic phosphate (Pi)} + H^+ \qquad (1)$$

$$\text{ADP} + \text{phosphocreatine (PCr)} + H^+ \leftrightarrows \text{ATP} + \text{creatine} \qquad (2)$$

The main source of ATP in heart muscle is oxidative phosphorylation of ADP in the respiratory chain. The enzymes of oxidative metabolism are located within the mitochondria. Because of the high energy turnover in heart muscle, the cell volume occupied by mitochondria in myocytes is greater than seen in most other tissues[5].

During ischemia, oxidative metabolism is inhibited and intracellular acidosis occurs (see 2,3 for review). A number of studies have demonstrated that the recovery of cardiac contractile function is impaired during reperfusion due to the presence of intracellular acidosis (see 1-3 for review). The major source of H^+ during anaerobic metabolism is the hydrolysis of glycolytically produced ATP[1-3]. The purpose of this thesis research was to re-evaluate this proposal and expand it, taking into account how glucose metabolism might influence H^+ production not only during ischemia but also during reperfusion. The fate of H^+ and its consequences on ion homeostasis were also considered.

1.1 Energy substrate metabolism

Cardiac contractile function is sustained by the hydrolysis of ATP produced mainly from the metabolism of both carbohydrates and fatty acids (Fig. 1-1). ATP production under normal aerobic conditions arises predominantly from the mitochondrial oxidation of acetyl coenzyme-A (CoA), derived from carbohydrates (primarily glucose and lactate), free fatty acids and to a lesser extent, ketone bodies and amino acids[6,7]. Glucose metabolism (glycolysis and glucose oxidation) is an important source of energy. Glycolysis has the advantage of producing ATP without the requirement of oxygen. Although glycolysis only contributes 5-10% of the overall ATP supply in the normal aerobic heart[7], it is well known that glycolytic ATP production has a special role in maintaining ion homeostasis within the myocyte[8,9]. The other major part of glucose metabolism is glucose oxidation, in which pyruvate derived from glycolysis is taken up by mitochondria and decarboxylated by pyruvate dehydrogenase (PDH) to form acetyl CoA, which is further metabolized by the mitochondria, eventually leading to ATP production. . The major source of intramitochondrial acetyl CoA arises from the β-oxidation of fatty acids.

Furthermore, as the contribution of fatty acid oxidation as a source of acetyl CoA production increases, the contribution of glucose oxidation as a source of acetyl CoA decreases[10].

1.1A. Fatty acid metabolism

Fatty acids represent the body's main fuel reserve. Because oxidation of long-chain fatty acids releases more than six times as much energy as the oxidation of an equal molar amount of glucose, and because glucose is readily converted into fatty acids, fatty acids represent a substantial, if not the predominant, fuel source for heart muscle[2,3].

Fatty acids are present in the blood either as unesterified (free fatty acids), or esterified as triacylglycerols, phospholipid or cholesterol esters. Albumin is a carrier protein in blood plasma, which increases the solubility of free fatty acids. Fatty acid esters (i.e. triacylglycerols) are carried in the blood by carrier molecules. Chylomicrons are triacylglycerol-rich particles formed by epithelial cells of the small intestine. Very low-density lipoprotein (VLDL) particles are also a source of fatty acids for the heart. These particles are synthesized in parenchymal liver cells. Once in the coronary circulation, fatty acids are released from chylomicrons and VLDL particles by the action of a vascular endothelium-bound enzyme, lipoprotein lipase (LPL). The fatty acids bind to albumin and are transported into the myocyte where they are used primarily as energy substrates.

The mechanism by which fatty acids enter the myocyte is not clearly defined. A group of membrane-spanning fatty acid transport proteins has been identified in heart muscle[11,12]. However, at high concentration, it is reported that fatty acids can passively enter the cell[12,13]. Once inside the cell, the majority of fatty acids enter the mitochondria to undergo β-oxidation. The activation of long-chain fatty acids to long-chain fatty acetyl-CoA esters occurs in the myocyte via an energy-dependent process catalyzed by long-chain fatty acyl-CoA synthetase, an outer mitochondrial membrane-bound protein. Activated long-chain fatty acyl-CoA can also be used for triacylglycerol synthesis in the heart. Fatty acyl-CoA, which is not shunted towards triacylglycerol synthesis, is transported into the mitochondria. This is achieved by carnitine-mediated translocation involving carnitine palmitoyltransferase I (CPT I), carnitine acyltranslocase, and carnitine palmitoyltransferase II (CPT II). Upon entry into the mitochondrial matrix, fatty acids undergo β-oxidation to yield acetyl-CoA, NADH and H^+, and $FADH_2$. Acetyl-CoA enters the tricarboxylic acid (TCA) cycle to produce further reducing equivalents for the electron transport chain, and ATP is subsequently generated (Fig 1-1).

The CPT I system is inhibited by physiologic concentrations of malonyl-CoA, the product of acetyl-CoA carboxylation, and was first characterized in the liver, where malonyl-CoA serves as a signal regulating the relative rates of fatty acid oxidation, ketogenesis, and triglyceride synthesis[14]. Although the heart is not a lipogenic organ, malonyl-CoA is present there[15], and the inhibition of cardiac CPT I by malonyl-CoA has been demonstrated[16]. Although there is no direct evidence, it is speculated that malonyl-CoA may play an important role in substrate competition between fatty acids and glucose. It is noteworthy that, in the isolated perfused working heart, glucose suppresses the oxidation of [^{14}C]octanoate, a short-chain fatty acid that does not require the CPT system for transport across the inner mitochondrial membrane[17]. It is possible that acetyl-CoA production from glucose oxidation may inhibit fatty acid oxidation (see 1.1C section for details). It is also noteworthy that ischemia reverses the inhibition of CPT I by malonyl-CoA, most likely through a modification in elaborate, thus making available increased amounts of palmitoyl-carnitine for possible oxidation on reperfusion[18].

1.1B. Glucose and lactate metabolism

Glucose uptake

One of the other major energy substrates for the heart is glucose. Generally, glucose is transported into myocytes by a facilitative transporter (GLUT 1), which may be responsible for basal glucose uptake. Insulin can increase glucose uptake by activating the translocation of GLUT 4 transporters from intracellular pools to the plasma membrane[19,20]. In normal heart muscle, cardiac work, the availability of alternate substrates as well as plasma glucose and insulin concentrations, act in concert as the most important factors regulating glucose uptake[21]. Ferrannini et al[22] found that hyperinsulinemia specifically enhances glucose uptake, converts fuel reliance from fatty acids to carbohydrate without changing O_2 consumption, and does not affect cardiac hemodynamics and external work in human heart. Myocardial glucose uptake is also determined by the dietary state[23], by O_2 availability[24-26] and by hormones that, in addition to insulin and glucagon, include catecholamines[27] and thyroid hormone[28]. The exact mechanisms by which the physiologic environment regulates glucose uptake are not yet clear. Once inside the cell, glucose is phosphorylated by hexose kinase and can then be shunted to glycogen synthesis or shuttled through glucose phosphate isomerase and into glycolysis.

Glycogen turnover

A storage form of glucose in the myocardium is glycogen. Glycogen is a large molecule with a branched structure and a molecular weight of about 107 kDa [29]. Although glycogen is more abundant in cardiac than skeletal muscle, the function of the large amount of glycogen in cardiac muscle (up to 2% of the cell volume) is not entirely clear[30]. Glycogen granules are abundant in the cytoplasm of cardiac myocytes throughout fetal development[31], and glycolysis is the predominant source of ATP production immediately after birth[32]. The high concentration of glycogen in fetal cardiac muscle may explain why the heart can maintain its contractile activity in the face of severe hypoxia[33]. The glucose preference of the newborn heart suggests that the fetal and newborn heart rely on glucose as the main substrate for energy production.

The control of glycogen metabolism is complex in that the synthetic pathway (involving glycogen synthase) and the degradative pathway (involving glycogen phosphorylase) are separate. The combined effects of protein phosphorylation and dephosphorylation on glycogen synthase and phosphorylase provide an interlocking system by which hormones (such as epinephrine) and mechanical activity (through Ca^{2+}) can control the net flux of glucose-1-phosphate into and out of the glycogen stores[34,35]. Epinephrine-induced cAMP formation promotes protein phosphorylation and simultaneously inhibits glycogen synthesis while stimulating glycogen breakdown, whereas stimulation of protein dephosphorylation by insulin shifts the balance toward glycogen synthesis.

Excess rates of glycogen breakdown during ischemia have been considered deleterious to the recovery of contractile function[36]. It has been reported that glycogen depletion and the attenuation of intracellular acidosis during ischemia are important factors in delaying irreversible injury and reducing infarct size in a rat model of myocardial preconditioning[3,37,38]. However, other investigators found the opposite: enhanced glycogen stores improved contractile function in the postischemic heart[39,40]. It is speculated that glycogen has a physiologic role apart from its role as fuel reserve that may involve its role as an "anchoring molecule" for other macromolecular cell constituents [41].

Glucose and lactate metabolism:

Glucose metabolism can be separated into two pathways (Fig.1-1,1-2). The first pathway involves the anaerobic catalysis (glycolysis) of one glucose molecule to two pyruvate molecules. The rate-limiting step of this pathway during aerobic perfusion of the heart is catalyzed by phosphofructokinase (PFK). During situations where oxygen supply is limited (ischemia or hypoxia), the enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) becomes rate-limiting[42]. Pyruvate from glycolysis can pass into a second pathway where it is metabolized by pyruvate dehydrogenase (PDH) to form acetyl-CoA for the TCA cycle. Alternatively, pyruvate can be reduced by NADH to form lactate, or be transaminated to form alanine or be carboxylated to form oxaloacetate. Lactate and most of the alanine are formed in the cytosol by near-equilibrium reactions, and both metabolites may be washed out from the cell. In well-oxygenated, working heart muscle, the bulk of pyruvate, however, enters mitochondria for oxidation. For every glucose molecule that passes through glycolysis to produce two pyruvate molecules, 2 ATP are produced.

Lactate and pyruvate can be transported into cell via a monocarboxylate carrier (MCT)[43] (see section 2.3 for details). Lactate is oxidized by lactate dehydrogenase (LDH) to form pyruvate and NADH and further utilized for energy production. The exact contribution of lactate to overall ATP production in the heart is a matter of controversy. Worth of mention, the inner mitochondrial membrane represents a barrier to charged molecules. Specific transporter mechanisms are required to enable transport of specific metabolites through the lipid bilayer. Monocarboxylate carrier of inner mitochondrial membrane is the transporter of pyruvate. Once pyruvate enters the mitochondria, it may undergo either decarboxylation to acetyl-CoA or carboxylation to oxaloacetate. The capture of metabolically produced CO_2 from the pyruvate dehydrogenase reaction to form acetyl-CoA provides an example for the efficient use of one substrate providing two precursors for citrate synthesis and efficient recycling of CO_2[44].

Regulation of glycolysis

Lack of O_2, clinically encountered in the context of ischemia, enhances flux through the glycolytic pathway that in turn augments substrate level phosphorylation of ADP and the formation of lactate. Other stimulants of flux through the glycolytic pathway are increases in cardiac work, short term exercise[45], hypertrophy[46] or sustained hypertension[47].

The catabolism of glucose-6-phosphate in the glycolytic pathway exhibits two major control sites: PFK and GAPDH. Allosteric control of PFK provides for large changes in catalytic activity: PFK activity is increased by insulin, ADP, AMP, Pi, fructose 1,6 bis-phosphate, fructose 2,6 bis-phosphate, cAMP, and ammonium ions. PFK activity is decreased by ATP, intracellular acidosis, PCr, and citrate (see 48,49 for review). During ischemia, GAPDH becomes rate-limiting for glycolysis. The activity of GAPDH is decreased by NADH and 1,3-

diphosphoglycerate, while insulin, AMP, ADP, glucose 1-phosphate and fructose 6-phosphate increase its activity[48].

Oxidative decarboxylation of pyruvate assumes a central position in the regulation of fuel supply to the heart. A system of intricate control mechanisms governs both activation and inactivation of PDH (Fig.1-3). Like most mammalian tissues, heart muscle possesses both active (dephosphorylated) and inactive (phosphorylated) pyruvate dehydrogenase, of which normally about 20% is in the active form[50]. The relative amount of active PDH (PDHa) increases with an increase in workload. PDHa may decline to only 1% to 5% of total PDH (PDHt) during starvation and in diabetes[51], that is, when noncarbohydrate substrates become the main fuel for respiration. Both PDH kinase and PDH phosphate phosphatase are probably active *in vivo*, and the relative proportion of active PDH must therefore be dependent on the relative activities of kinase and phosphatase as well as the intramitochondrial concentration of the effectors of these enzymes.

PDH, which irreversibly commits glucose-derived carbon to oxidation by the TCA cycle, is highly regulated in oxidative heart tissue (Fig.1-3). During reperfusion, heart muscle is exposed to high levels of fatty acids[52,53]. Increased plasma free fatty acid concentrations promote increased intramitochondrial fatty acid β-oxidation, raising acetyl-CoA/CoA and NADH/NAD^+ ratios. This activates PDH kinase, which phosphorylates and inactivates PDH (see 54 for review).

PDH is also subject to feed-forward regulation whereby increased concentrations of glycolytically-derived or LDH-derived pyruvate inhibit PDH kinase and thus activate PDH by allowing the PDH phosphatase activity to predominate[55,56]. Another mechanism of regulation of PDH in the heart is activation by increased workload or β-adrenoceptor agonism[57-59], which facilitate provision of energy from carbohydrates for contractile work. This is probably mediated by an increase in mitochondrial Ca^{2+} concentration, thereby activating PDH phosphatase and raising PDH activity[57-59].

1.1C. Substrate competition

Because of the omnivorous nature of the heart, glucose, lactate, fatty acids, ketone bodies are converted to acetyl-CoA and compete for the fuel of respiration. The relative predominance of one fuel over another depends on the arterial substrate concentration (which, in the case of fatty acids, ketone bodies, and lactate, can vary over a wide range), on hormonal influences, on workload, and on O_2 supply[6]. Although fatty acid oxidation can be almost completely suppressed when ketone bodies are abundant[60], there is a constant rate of carbohydrate use. The need for glucose or lactate is most likely due to the need for pyruvate carboxylation and anaplerosis to replenish the TCA cycle intermediates[60]. This is because pyruvate (from glucose or lactate) can generate both acetyl-CoA and oxaloacetate for the citrate synthase reaction, while fatty acids and ketone bodies can only provide acetyl-CoA (Fig.1-4). It has been shown that lactate at the relatively high concentration of 40 mM suppresses glucose uptake by 90% in the isolated working rat heart, whereas β-hydroxybutyrate at the same concentration suppresses glucose uptake only by 64%[61]. These findings suggest that the fuels for cardiac energy metabolism can be grouped into essential fuels, which provide both acetyl-CoA and oxaloacetate and include glucose, lactate, pyruvate and into nonessential fuels, which provide only acetyl-CoA and include fatty acids of all chain lengths, ketone bodies and amino acids[60].

Fatty acids are the preferred fuel for respiration in the fasted state[62], but even in the presence of high concentrations of fatty acids or ketone bodies, a certain amount of glucose continues to be oxidized[63]. Thus, glucose oxidation appears to be important for cardiac function. From the studies of Randle et al[64,50], we know that there is a hierarchy of fuel selection: the oxidation of fatty acids suppresses glucose oxidation (through inhibition of PDH). Almost 20 years later, Taegtmeyer et al[63] showed that the reverse is also the case: glucose suppresses the oxidation of long-chain fatty acids. Furthermore, Lopaschuk et al[52] have shown that fatty acid levels in the blood are markedly elevated after myocardial ischemia. Many factors could account for these high levels of plasma fatty acids, including endogenous and exogenous catecholamine release, heparin administration, cardioplegic agents, and the volume or metabolic status of the patients. High rates of fatty acid oxidation inhibit glucose oxidation, which results in a marked imbalance between glycolysis and glucose oxidation[65]. This is associated with poor recovery of cardiac function. Although the importance of the interaction of the two types of substrates is not completely understood, some of the metabolic signaling mechanisms, especially the inhibition of fatty acid oxidation by malonyl-CoA, are currently under intense investigation[66].

Many factors contribute to the selection of energy-providing fuels for the heart. According to Krebs et al[67], these factors may be classified under the following three main categories: 1) concentration of the fuel in the tissue; 2) the presence of the enzymes required for degradation; 3) the kinetic properties of the key enzymes, especially of those that initiate the release of energy. Each of these three main factors depends on a variety of components. The entry of fuels into the cell, as well as synthesis and degradation of stored fuel reserves, is controlled by hormones such as insulin and epinephrine and by other environmental factors, with cAMP and a cascade of intracellular signals acting as second messengers.

1.2. Source of H^+

During ischemia, when the supply of O_2 becomes limiting for oxidative phosphorylation, both fatty acid and carbohydrate oxidation decrease and ATP production is impaired. Glycolysis and glycogenolysis initially increase in an attempt to compensate for this decrease in ATP supply. Anaerobic glycolysis, while potentially beneficial due to its ability to generate ATP in the absence of O_2, may also be detrimental. During severe ischemia the beneficial effect of glycolysis is overshadowed by the accumulation within the myocardial cell of glycolytic by-products, particularly H^+ and lactate[65,68,69].

While considerable debate has focused on the potential benefits or harm associated with glycolysis uncoupled from glucose oxidation during ischemia, relatively little attention has been paid to the importance of this coupling during the critical period of reperfusion.

When glucose is metabolized by glycolysis followed by oxidation, with the associated synthesis and hydrolysis of ATP, the net production of H^+ is zero. This is because the extra H^+ derived from ATP produced by glycolysis combines with pyruvate and is transported into the mitochondria via the monocarboxylate carrier (see 70 for review) and is consumed in the TCA cycle to produce NADH, which is further utilized for ATP production. However, if glycolysis is uncoupled from glucose oxidation such that the rate of glycolysis exceeds that of glucose oxidation, there is a net production of two H^+ per molecule of glucose that passes through glycolysis that is not oxidized[8,65] (see following equation).

H^+ production from glucose metabolism:

1. Glycolysis + aerobic disposal of pyruvate

   glucose $\rightarrow 6\ CO_2 + 6\ H_2O$

2. Glycolysis + no aerobic disposal of pyruvate

   glucose $\rightarrow 2$ lactate$^-$ $+ 2\ H^+$

H^+ production rate = 2 × (glycolysis rate- glucose oxidation rate)

In the presence of high levels of fatty acids, which preferentially inhibit glucose oxidation, the uncoupling of glycolysis and glucose oxidation, even in the absence of ischemia, is a major source of H^+ production[8,65,71]. In most clinical situations of reperfusion, the heart muscle is exposed to high levels of fatty acids and this is associated with poor recovery of cardiac function[52,53]. If hearts are aerobically reperfused after ischemia, fatty acid oxidation quickly recovers to rates that can equal or exceed the preischemic rate[7,72,73]. The fatty acid oxidation rate during reperfusion of ischemic hearts is high due to a "dysregulation" of this pathway. Previous studies have shown malonyl CoA, a potent inhibitor of CPT I[74], is a key factor regulating changes in fatty acid oxidation in the postischemic heart[75,76]. This occurs due to a decrease in malonyl CoA production by acetyl CoA carboxylase (ACC), which is inhibited by 5'-AMP-activated protein kinase (AMPK)[75-77]. During reperfusion AMPK is activated, while ACC is inhibited. A decreased production of malonyl CoA occurs, resulting in an acceleration of fatty acid oxidation[75-78]. High rates of fatty acid β-oxidation dramatically inhibits glucose oxidation[65,72], which results in a marked imbalance between glycolysis and glucose oxidation. Therefore, uncoupling of glycolysis from glucose oxidation, which is enhanced during ischemia, persists during reperfusion. The resulting continued production of H^+ may maintain intracellular acidosis developed during ischemia and slow the recovery of intracellular pH (pH_i) during reperfusion. This may explain the depressed cardiac contractile function that has been noted during reperfusion[72,78]. The poor recovery of cardiac function in postischemic hearts may occur either by a direct action of acidosis on the contractile apparatus[79,80] or indirectly via an increased consumption of ATP for the correction of the associated ionic imbalances[81,82]. Thus, while a minimal level of glycolysis is essential for cell survival, excessive rates of glycolysis can be detrimental. Although the detrimental effects of intracellular acidosis generated from uncoupled glucose metabolism during ischemia are well demonstrated, no studies to date have directly assessed the actual contribution of H^+ production from glucose metabolism to the recovery of cardiac efficiency (Cardiac work/MVO_2) and rates of recovery of pH_i during reperfusion.

2. Fate of H^+ and cardiac contractile function

To maintain contractility, the cardiac cell needs to regulate its pH_i closely, considering the dramatic effects of changes in pH_i on ionic conductances, metabolic processes, Na^+ and Ca^{2+} homeostasis, and myofilament Ca^{2+} sensitivity[79,80,83]. In cardiac cells, as in any given cell, steady-state pH_i is strictly maintained within a narrow range at relatively alkaline values (7.1-7.2). To regulate its pH_i, the mammalian cardiac cell possesses at least five membrane ionic transporters. The activation of the Na^+/H^+ exchanger (NHE)[43], the Na^+-HCO_3^- co-transporter[44], the lactate-H^+ co-transporter[84], or the vacuolar-H^+ ATPase[87] induces intracellular alkalinisation, while activation of the Cl^-/HCO_3^-

exchanger triggers acidification[88]. Among these different pH regulating ionic transporters, the NHE has been the most studied in various tissues including the heart. This is because NHE plays an important role in the regulation of intracellular Na^+ and Ca^{2+} levels during reperfusion (see the following section). The physiologic significance of other H^+ extrusion pathways is less clear. However, the activities and relative importance of each of these H^+ extrusion pathways may be influenced by: 1) level of expression of the protein, 2) individual ionic transmembrane gradients, 3) regulation in response to a number of neurohumoral and transduction factors, and 4) drug-induced alteration of activity. The mechanisms for regulating the fate of myocardial H^+ may assume greater importance in the etiology of mechanical dysfunction under conditions of high levels of fatty acids, that enhance H^+ production due to inhibition of glucose oxidation and subsequent impairment of the coupling of glycolysis to glucose oxidation. Such conditions are present clinically and were mimicked in the present studies (see Chapter 2).

2.1 Roles of NHE in regulation of pH_i and the recovery of cardiac mechanical function in postischemic hearts

NHE plays a critical role in the regulation of pH_i by removing H^+ that are continuously generated during ischemia and reperfusion. NHE1, the so-called housekeeping isoform, is ubiquitously distributed in most tissues and is the primary NHE subtype found in the mammalian cardiac cell[89-91]. There are at present five known NHE isoforms in the plasma membrane of mammalian cells[89-91]. The function of NHE is the regulation of pH_i and cell volume through extrusion of H^+ in exchange for Na^+ influx in a 1:1 stoichiometric relationship, rendering the process electroneutral[92]. Although NHE is a major regulator of pH_i it may share this function with a number of other cellular homeostatic pH regulatory systems, as referred to above. Whereas the localization of the other NHE isoforms appears to be restricted to the plasma membrane, the possibility that NHE6 is a mitochondrial isoform suggests that this subtype may regulate mitochondrial function particularly with respect to intramitochondrial Na^+ and H^+ levels[93]. This also suggests that NHE6 may play an important role in the regulation of energy metabolism.

2.1A. Structure and localization of NHE1

Fig. 1-5 represents the putative topological model for the mammalian NHE1, which consists of the following 2 principal domains: a 500–amino acid transmembrane domain and a 315–amino acid highly hydrophilic carboxyl-terminus cytoplasmic domain. The number of membrane-spanning units differs according to NHE isoform type. NHE1 contains 12 such spanning regions that are critical for the maintenance of NHE1 function in terms of H^+ extrusion. The hydrophilic cytoplasmic region plays an important role in modulation of the exchanger, especially through phosphorylation-dependent reactions[94-96]. Although the predicted molecular mass of NHE1 is 91 kDa, the protein is glycosylated and its apparent molecular mass is 110 kDa.

A recent study suggested that NHE1 is localized primarily in the intercalated disk region of atrial and ventricular myocytes in close proximity to connexin 43 and, to a lesser degree, at the transverse tubular system[97]. The significance of such localization of NHE1 is not clear, but it may implicate the exchanger in cell-to-cell ion-dependent communication via gap junctions, as well as the regulation of $[Ca^{2+}]_i$ levels through proton-dependent modulation of Ca^{2+} channels in transverse tubules.

2.1B. Regulation of NHE1 activity

Intracellular acidosis is the major stimulus for NHE1 activation[98]. However, its activity is inhibited by increased extracellular H^+, by analogues of amiloride including dimethylamiloride (DMA) or newly investigated agents such as HOE694, HOE642 (cariporide) or EMD 85131[99-101]. Intracellular Na^+ ($[Na^+]_i$) is unlikely to be a major regulator within the physiological range (7 to 16 mM)[98], although about a 10-fold concentration difference across the membrane occurs ($[Na^+]_o/[Na^+]_i = 142/16$). The NHE1 has a steep relationship between the internal pH and activity[90]. At low pH_i ($pH \leq 6.5$), the exchanger is maximally active. The Hill coefficient of activation in the myocardium is near 3, steeper than in some other cell types[90]. This results in maximal activation over a narrow pH range[102].

Phosphorylation of NHE1 by protein kinases

The regulation of NHE isoforms by various kinase pathways has been studied in both native tissues as well as in tissues deficient in endogenous exchanger activity. Pouysseyur's group first demonstrated that the NHE1 is a phosphorylated glycoprotein under *in vivo* conditions[103]. Increased phosphorylation was observed after stimulation by thrombin or EGF and was associated with cytoplasmic alkalization, suggesting enhanced Na^+/H^+ exchange. Phosphorylation was restricted to serine residues, and purification and trypsin digestion of NHE1 yielded similar phosphopeptides for both stimuli[104].

Phosphopeptide mapping of NHE1 and expressed deletion mutants revealed that the major phosphorylation sites all mapped to the cytoplasmic segment, while the transmembrane segments do not appear to be phosphorylated[105,106]. Several isoforms of NHE have been found to be activated or inhibited by stimulation of tyrosine kinase receptors (see 90,107 for review). Stimulating protein kinases PKA, PKC or PKG either directly or via upstream receptors coupled to heterotrimetric G-proteins will also regulate NHE1 activity[107]. This includes receptors for β-adrenoceptor agonists which are coupled to protein kinase A, angiotensin II and several drugs acting on PKC, and atrial natriuretic peptide (ANP) which leads to stimulation of PKG[107]. PKG seems to be essential for the action of ANP on NHE since cGMP, generated from GTP by particulate guanylate cyclase coupled to the ANP receptor, has no direct effect on Na^+/H^+ exchange, in contrast to an apical Na^+ channel which is directly regulated by cGMP[108].

Calcium/calmodulin (CaM)

It has been shown that NHE1 itself is also a Ca^{2+}/CaM-binding protein (see 90, 107 for review) and direct phosphorylation of NHE1 by Ca^{2+}/CaM/PK II has been shown using an *in vitro* phosphorylation assay[109]. Binding experiments with CaM-sepharose and dansylated calmodulin[69] showed that NHE1 binds CaM in a strictly Ca^{2+}-dependent manner. Two different binding sites, one with a high affinity (Kd~20 nM) and one with a relatively low affinity (Kd~350 nM), could be identified and located to amino acids 636-656 (termed region A), and amino acids 664-684 (termed region B), respectively[109,110]. These regions are rich in positively charged amino acids (Arg, Lys, His) and almost completely lack negatively charged amino acids (Asp or Glu), a common feature of many calmodulin binding proteins. The high affinity CaM-binding region A is involved in the activation of NHE1 in response to growth factor and osmotic stress, the direct binding of Ca^{2+}/CaM to region A may be a key event in the Ca^{2+}-involved activation of NHE1. It is hypothesized that CaM-binding region A functions as an "autoinhibitory domain" and that Ca^{2+}/CaM activates NHE1 by binding to region A and thus abolishing its inhibitory effect[106].

ATP dependence of NHE1

In all cases studied to date, the exchange reaction appears to be reversible and driven solely by the transmembrane chemical gradients of H^+ and Na^+ (H^+ gradient is the major force). Transport is generally believed to be passive, not requiring expenditure of metabolic energy. However, it is now well established that in intact cells depletion of ATP induces a marked depression of the rate of exchange[111,112]. The mechanism whereby ATP modulates NHE1 remains obscure. Although speculative, it is possible that very low ATP levels in the ischemic myocytes counter the stimulatory effect of intracellular acidosis on NHE1 activity.

H^+ sensor

It is well known that intracellular acidosis is the major activator of NHE1. However, the detailed mechanisms regarding H^+ activation of NHE1 is not clear. It has been suggested that approximately 60% of proton-removal capability of the cardiac cell is accomplished via NHE1[113]. NHE1 activity is regulated by hormones, paracrine/autocrine regulators, and mechanical stimuli such as hyperosmotic challenge, mostly via phosphorylation reactions[99], resulting in increased affinity of the so-called H^+ sensor[105,106] (Fig. 1-5). Although the nature of this sensor, which is responsible for the exquisite sensitivity of NHE1 to changes in pH_i, is poorly understood, there is evidence[100] that H^+ binding triggers a protein conformational change within an NHE1 oligomer resulting in NHE1 activation. The sensitivity of the H^+ sensor is mainly regulated by distinct regions of the C-terminal, and agonists that activate NHE1 via phosphorylation reactions shift the pH_i -NHE1 activity curve toward the alkaline range, that is, there is greater activity at less acidic pH_i. It is thought that somewhere between amino acids 515-595 reside the amino acids required for maintenance of "normal" intracellular H^+ sensitivity[114].

2.1C. NHE1 activity in ischemia and reperfusion

The contribution of NHE1 to pH_i regulation under normal aerobic conditions is controversial as a number of studies indicate that the system does not operate when pH_i is within normal limits. Undoubtedly, NHE1 plays an important role in the regulation of pH_i and cardiac function in the ischemic and reperfused myocardium. Ischemia-induced acidosis represents the major stimulus for NHE1 activation with further stimulation by phosphorylation-dependent processes. In addition to activity, NHE1 mRNA levels are also increased in both the ischemic myocardium as well as in hearts exposed to cardiotoxic ischemic metabolites,[115,116] including lysophosphatidylcholine[116], suggesting that a cardiac insult increases NHE1 expression. There are extensive data showing that NHE1 has a critical role in the postischemic heart. During reperfusion, extracellular pH quickly recovers, while pH_i remains low. This results in a H^+ gradient across the plasma membrane and NHE1 is hyperactivated. Efflux of H^+ by NHE1 is rapid and is accompanied by increases in intracellular Na^+. Since Na^+-K^+ ATPase is inhibited due to ATP depletion during ischemia, the accumulation of Na^+ leads to Ca^{2+} overload via Na^+/Ca^{2+} exchange (NCE)[117-120].

The cardiac NCE mediates the counter-transport of 3 Na^+ for 1 Ca^{2+} across the sarcolemmal membrane. It is a bidirectional transport process, capable of moving Ca^{2+} in either direction across the sarcolemma, depending on membrane potential and the transmembrane gradients of Na^+ and Ca^{2+}. There are 2 possibilities for the mode of operation of the NCE during ischemia/reperfusion[121]. First, NCE may continue in the normal, Ca^{2+} efflux, mode during ischemia[68]. In this mode, accumulation of intracellular Na^+ due to activation of NHE slows down intracellular Ca^{2+} efflux, resulting in intracellular Ca^{2+} overload. Second (reverse mode), NCE could operate in the Ca^{2+} influx mode during ischemia/reperfusion[121], leading to increased intracellular Ca^{2+}. Under this condition, intracellular Na^+ efflux due to huge accumulation of Na^+ triggers Ca^{2+} influx during ischemia/reperfusion. NCE involvement in ischemia and reperfusion injury has recently been suggested by the finding that such injury is enhanced in hearts of mice overexpressing this exchanger[121]. The fact that injury is exacerbated in mice that overexpress NCE could also be taken to suggest that this exchanger actively participates in injury possibly by increasing Ca^{2+} influx through a reverse mode process.

It has been suggested[122] that activation of the NHE1 at the time of reflow represents a major component of reperfusion injury. This would contribute to restoration of pH_i. On the basis of the concepts discussed in the preceding section, the concomitant Na^+ influx would increase $[Ca^{2+}]_i$ through the NCE.

Surprisingly, there is still uncertainty as to whether inhibiting NHE1 during ischemia accenuates intracellular acidosis. One study showed that the NHE1 inhibitor, DMA, resulted in greater acidification during ischemia and slower recovery from acidosis after reperfusion[122]. Although this is strongly suggestive for a role of NHE1 in the regulation of pH_i during ischemia *per se*, other investigators have failed to demonstrate this effect with ethylisobutylamiloride or HOE642 despite the ability of these drugs to attenuate Na^+ loading and improve ventricular recovery[123]. This may have arisen because of the ability of other pH-regulatory processes, such as the Na^+-HCO_3^- exchanger or the lactate-H^+ co-transporter, to compensate for NHE1 inhibition. It remains controversial whether activation of NHE1 occurs during ischemia or during reperfusion or both. There are two possible mechanisms that may contribute to the above discrepancy: 1) Different degrees of ischemia may differentially affect NHE1 activity. During mild ischemia, NHE1 may be activated due to less extracellular H^+ accumulation. In contrast, during severe ischemia, NHE1 could be inhibited due to extracellular H^+ overload. It is not clear whether lactate itself, one of the major metabolic products of ischemia, affects NHE1 activity. 2) Intracellular ATP levels play an important role in the maintenance of activity of NHE1[111,112]. Although NHE1 mediated flux of Na^+ and H^+ across the membrane is a passive process, depletion of intracellular ATP content has been shown to inhibit its activity[111,112]. Thus, during severe ischemia, the dramatic decrease in ATP may inhibit NHE1 activity. Therefore, inhibition of this exchanger may not affect the concentrations of intracellular Na^+, Ca^{2+} and H^+. In contrast, NHE1 activity may be more active during reperfusion due to partial recovery of ATP production. However, the role of the extent of recovery of ATP production and content on NHE1 activity has not been addressed.

A number of experimental and clinical studies have demonstrated that inhibition of NHE1 can significantly improve cardiac function after ischemia. As summarized in the Table 1, these effects include enhanced contractility, reduced hypercontracture, and a decrease in the incidence of arrhythmia. Cariporide, a potent NHE1-selective inhibitor, exerts dramatic protective effects in various experimental models and in terms of numerous parameters under both *in vitro* and *in vivo* situations. This may explain the relatively rapid development of this drug for clinical use as a cardioprotective strategy. Likewise, the benzoylguanidine compound EMD 85131 has recently been shown to exert potent infarct-reducing effects in a canine occlusion-reperfusion model, which has also resulted in its entry into the clinical arena[124].

Recently, the GUARDIAN (Guard During Ischemia Against Necrosis) study, a Phase II/Phase III double-blind, randomized placebo-controlled study of >11,500 patients, assessed different doses of cariporide (HOE642) in individuals with acute coronary syndromes[125]. This study failed to demonstrate an overall significant attenuation (10%) of the 2 primary events, mortality and incidence of myocardial infarction; however, favorable effects among the 3 major subgroups were observed in those patients receiving the highest dose (120 mg every 8 hours) of the drug, including a significant event rate reduction in high-risk patients undergoing coronary artery bypass surgery. These results are therefore encouraging, especially given that the study also represented a dose-finding component, and overall support the concept that NHE1 inhibition represents a safe, therapeutic approach for cardioprotection that undoubtedly deserves further attention.

Studies by Maddaford et al[126] have confirmed cardioprotective properties of DMA and it has been reported that DMA improves cardiac efficiency without altering glucose metabolism[78]. Thus, manipulation of the fate of H^+ via NHE pathway affects the recovery of mechanical function independent of H^+ production from glucose metabolism.

2.2 Monocarboxylate carrier (MCT) and coupling of glucose metabolism

Efflux of H^+ may also occur by MCT that is stimulated by intracellular H^+ and lactate overload produced by glycolysis[86]. Efflux of H^+ by MCT may have a potential advantage since the deleterious consequences of Na^+ accumulation are avoided. Studies by Wang et al[127] have demonstrated the existence of two distinct MCT subtypes in heart cells (MCT1 and MCT2), with both of these carriers being present in the same myocyte. Both transporters have a stoichiometry of 1 lactate: $1H^+$. Three important conclusions can be drawn from characterization of MCT in the heart cell membrane: 1) MCT is able to transport

L-lactate acid into and out of the heart at rates sufficient to keep pace with the normal requirements of the heart. However, in the working heart using lactate as a major energy source, the transporter may operate close to its maximal capacity, and under extreme conditions, such as during hypoxia and severe ischemia, the carrier may actually limit the rate of lactic acid efflux from the heart[127]. In the hypoxic heart, the rate of lactate efflux can significantly exceed calculated maximal rates of lactate efflux by the carrier[86]. Presumably, any efflux of lactate in excess of that catalyzed by the carrier must occur by free diffusion under this condition. 2) The relatively low K_m value of the carrier for pyruvate (<0.2 mM) may explain why pyruvate efflux from the perfused heart occurs at a rate faster than L-lactate (K_m=2 mM) at the same intracellular concentration[128]. The physiologic significance of this is not clear. 3) Of importance, the presence of MCT in mitochondria enables rapid entry of pyruvate (instead of lactate) and H^+ into the mitochondria when they are to be oxidized (see 129 for review). This is very important for glycolysis coupling with glucose oxidation since 2 H^+ produced from glycolysis combined with 2 pyruvate enter the mitochondria and are consumed by the TCA cycle to produce NADH, which is further utilized for ATP production.

In ischemia, the rate of glycolysis increases as the heart seeks to maintain tissue ATP concentrations to drive contraction and preserve ionic homeostasis. In severe ischemia, the lack of blood flow does not allow the lactic acid that is transported out of the cell to be carried away. Thus, inhibition of net lactic acid efflux from the cell rapidly ensues, because efflux and influx rates soon equilibrate. Meanwhile, pyruvate can not be oxidized and is therefore converted into lactate via LDH (glycolysis uncoupled with glucose oxidation) within the cytoplasm. As lactate accumulates within the cell, it may itself have detrimental effects on ionic homeostasis and heart function,[130-132] but the greater effect is through the concomitant buildup of H^+ and consequent decrease in pH_i. This is known to inhibit glycolysis and hence limit further ATP production, as well as having a direct effect on the contractile machinery[133,134].

During reperfusion, pH_i returns to normal through the operation of H^+ extrusion pathways. Because lactic acid efflux from the cell is a necessary consequence of increased glycolysis, it would seem likely that MCT may also be regulated in this manner. It is clear that heart cells contain at least 2 MCT isoforms, MCT1 is confined to the intercalated disk region[88] (similar to NHE1) and is used to transport lactate, pyruvate into cells for oxidation and between cells through the intercalated disks. Other MCT isoforms may be more important for the loss of lactate and H^+ during ischemia and reperfusion. It will be interesting for future studies to identify the "heart specific" isoform and characterize its kinetic properties and substrate specificity in order to see how these may relate to the physiologic and pathologic function of the heart and cardiac glucose metabolism.

2.3 Na^+-HCO_3^- co-transporter

A Na^+- and HCO_3^- - dependent transporter is present in cardiac myocytes that increases pH_i by neutralizing intracellular protons[85,135]. However, as in the case of NHE1, activation of this system may also cause Na^+ and hence Ca^{2+} overload. Previous studies have shown that it is involved in the regulation of myocardial pH_i[136], but it may have a lesser role in the regulation than NHE1. It is estimated that in normal myocytes, the contribution of the total efflux of H^+ equivalents by the NHE1 and of the Na^+-HCO_3^- co-transporter carriers to be 69% and 31%, respectively, at pH_i 6.90, and 67% and 33%, respectively, at pH_i 6.75[85]. It is possible that Na^+-HCO_3^- co-transporter may play a greater role in the regulation of pH_i in the presence of NHE1 inhibition. A recent study has shown that simultaneous inhibition of NHE1 and Na^+-HCO_3^- co-transporter is needed to protect myocardial cells against reoxygenation-induced hypercontracture[137]. That suggests that inhibition of this co-transporter may attenuate Na^+ and subsequently Ca^{2+} influx.

2.4 Cl^--HCO_3^- exchanger (anion exchanger, AE)

AEs mediate the transmembrane flux of the anions, Cl^- and HCO_3^-. The AE family is comprised of at least three members, AE1, AE2, and AE3[138]. Although extensively studied in a number of cell types, little is known about the role and regulation of the cardiac isoform, AE3[88]. AE activity may cause acidification or alkalization dependent on the concentration gradients of HCO_3^- and Cl^- [139]. The Cl^- gradient (Cl^-_o/Cl^-_i =105/50) favors Cl^- influx, HCO_3^- efflux and hence cytoplasmic acidification. However, indirect evidence suggests that AE3 in contracting hearts may cause alkalization via HCO_3^- influx[139].

The critical role for the CO_2/HCO_3^- buffer system for pH homeostasis is dependent on carbonic anhydrase (CA), an enzyme that catalyses the hydration-dehydration of CO_2/HCO_3^- [140]. The CA isoform present in the heart is CAIV[141], which is linked extracellularly by a glycosylphosphatidyl inositol anchor to the plasma membrane[142]. CAIV has an important role in facilitating the recovery of pH_i after reperfusion of ischemic heart[141]. This is because freely diffusible CO_2 may rapidly move out of the cell and be converted to HCO_3^- by CAIV. The extracellular HCO_3^- can then be exchanged for intracellular Cl^- by AE3 proteins, leading to cellular alkalization. Thus, under conditions of mechanical work and high level of glucose and fatty acid oxidation which generates large amount of CO_2, AE3 activity would be expected to be beneficial by reducing intracellular acidosis and the potential for Na^+ accumulation by NHE1.

2.5 Vacuolar-H^+ ATPase

Recently, a newly identified H^+-extruding process, vacuolar-H^+ ATPase, was found to operate in cardiomyocytes[143]. Vacuolar-H^+ ATPase has been described in a variety of cell types, being responsible for the acidification of intracellular compartments, such as synaptic vesicles, lysosomes, and endosomes[144]. Vacuolar-H^+ ATPase has been detected in the plasma membranes of some types of cells, thereby maintaining the cytosolic pH by extruding H^+ out of the cells[144]. Previous studies also show that vacuolar-H^+ ATPase may play an important role in cardiomyocyte protection during ischemia by reducing intracellular acidosis, which can prevent apoptosis and NHE1-induced Ca^{2+} overload[143]. It is possible that vacuolar-H^+ ATPase mediated H^+ efflux may spare Na^+ overload via NHE1 and thereby attenuate Ca^{2+} influx via NCE. Evidence supports the hypothesis that acidosis itself contributes to apoptosis. There are also reports showing that (1) intracellular acidification precedes apoptosis in neutrophils[145] and Jurkat cells[146] (2) apoptotic cells have lower pH_i compared with normal cells[147] and (3) acidic endonuclease (DNAse II), which becomes active at pH 6.8 and below, is involved in apoptosis[148]. However, a remaining question is whether activation of vacuolar-H^+ ATPase improves the recovery of cardiac mechanical function and cardiac efficiency in postischemic hearts. The relationships between vacuolar-H^+ ATPase and other H^+ extrusion pathways which contribute to the regulation of pH_i are unknown.

3. Determination of pH_i by ^{31}P-NMR spectroscopy

The method of measuring pH_i through ^{31}P-NMR has become invaluable to cardiac research because it offers unique opportunities to examine the pH of the heart noninvasively. To help in understanding this method, the following provides a brief background.

NMR arises as a consequence that certain nuclei have an odd number of protons giving them what is known as *net nuclear spin*. Some examples of nuclei with net spin are ^{1}H, ^{2}H, ^{15}N, ^{17}O, ^{31}P, ^{13}C and ^{23}Na. Because the nucleus is charged, its spin causes a small magnetic field producing a *magnetic moment*, meaning that they behave like a magnet that spins around its magnetic axis. When a nucleus with a non-zero magnetic moment is exposed to a constant magnetic field, it tends to align its magnetic axis preferentially with this field but its rotation prevents a straightforward alignment of the magnetic axis. The overall result is analogous to a spinning top that opposes reorientation of its axis and begins to wobble. In other words, the nuclear magnetic axis begins to process with a frequency that is characteristic for each nucleus.

It is possible to irradiate the sample with an alternating electromagnetic field in the radiofrequency (RF) range of typically 10-100 MHz (depending on the nucleus and the magnet's field strength). When the RF frequency is identical to the precession frequency, resonance occurs. A small fraction of nuclei in their ground state absorb energy then and are excited to the higher state. When the RF pulse is ended, the ratio of excited to ground state nuclei returns to what it was before. This return can be monitored electronically, again through RF circuitry. A mathematical (Fourier) analysis of the time course of this decay--free induction decay (FID) provides the information of the resonance frequencies of the nuclei.

The purpose in NMR is to determine the exact resonance frequency of the nuclei and the intensity at each frequency. NMR provides information about the chemical environment of the nucleus because, as part of an atom, the nuclei is surrounded by electrons which partly shield the imposed magnetic field. Thus the effective local magnetic field, B_L, sensed by the nucleus is altered compared to the external applied field, B_o:

$$B_L=B_0(1-\delta)$$

where the correction δ, referred to as the shielding tensor, is independent of B_o but is only a function of the chemical/electronic environment of the nucleus.

Therefore, two identical nuclei with different chemical environments will experience different B_L's and can be shown to have different positions (*chemical shift*) in the NMR spectrum, caused by their chemical environment. The chemical shift is normally given as the fraction, in parts per million or ppm, of the reference frequency derived from a standard compound. The advantage of this frequency normalization is that the frequency scale of the NMR spectrum becomes independent of the field strength of the NMR machine.

Measurement of cellular pH is mainly based on an analysis of the cellular phosphate content. In living systems Pi exists predominantly as either HPO_4^{2-} or $H_2PO_4^-$. The rate of equilibration between these two forms is much more rapid than the nuclear precession frequency. Therefore, the two phosphate forms are only visible as one resonance, the frequency of which depends on the pH_i[149].

Typically the calculation of pH_i uses the chemical shift of the Pi peak from ^{31}P spectra. Since the PCr signal does not change with pH_i, it is a useful reference to determine precisely the pH-dependent shifts of the phosphate peaks.

Pi is not the only nucleus available for measuring cytoplasmic pH. The 2,3-diphosphogluconate (2,3-DPG) is also a good candidate[149]. However, the signals emanating from Pi and from 2,3-DPG can overlap to a significant degree, making the spectrum in this region uninterpretable. Red blood cells contain significant quantities of 2,3-DPG, which means that circulating blood interferes with measurements of the intracellular phosphate signal. It is therefore impossible to measure reliably the pH_i of the heart *in vivo*. That is the major reason that the isolated working heart, perfused with oxygenated crystalloid solution instead of blood, was used in this study. To overcome further contaminating resonances, it was important to reduce or eliminate the concentration of Pi in the perfusion medium. In the present studies, KH_2PO_4 was omitted from the Krebs-Henseleit solution.

4. Targets for pharmacotherapy: Effects of alteration of energy metabolism on pH_i and postischemic recovery of cardiac function

Intracellular acidosis is one of the major triggers for ischemia and reperfusion injury. During reperfusion, NHE1 is hyperactivated and plays an important role for the recovery of cardiac function and regulation of pH_i in postischemic hearts. It is estimated that H^+, which are continually generated from uncoupled glucose metabolism during reperfusion[65,72,78], is the one of the most important activators for NHE1. Reducing H^+ production during reperfusion by improving coupling of glucose metabolism has been shown to improve the recovery of cardiac function and efficiency[72,78]. As the increases in intracellular Na^+ and Ca^{2+} levels that arise after activation of the NHE1 and NCE require ATP to restore ion homeostasis[81,82], either inhibition of NHE1 or reducing H^+ production may lead to an improved efficiency in the utilization of energy for contractile work. Furthermore, previous studies have shown that inhibition of NHE1 improves the recovery of cardiac efficiency and preserves PCr content during reperfusion[81,82]. Control of pH_i by Na^+-independent systems offers the advantage of reducing acidosis without causing adverse effects on Na^+ gradients. The present studies focus on both the source and fate of H^+ on the recovery of mechanical function and cardiac efficiency during reperfusion after ischemia.

High plasma fatty acid concentrations have been shown to increase the severity of ischemic damage in a number of different experimental animal models of cardiac ischemia, and has also been linked to a depression of mechanical function during reperfusion in postischemic hearts[65,72,78]. It is estimated that intracellular H^+ generated from uncoupled glucose metabolism, due to the presence of high levels of fatty acids, contributes to the detrimental effects of high levels of fatty acids on cardiac contractile function during reperfusion.

A number of different approaches can be used to manipulate energy metabolism in the heart. One way to increase glucose metabolism and decrease fatty acid metabolism in the heart is to decrease circulating fatty acid levels. A decrease in the level of fatty acids will improve the coupling of glycolysis to glucose oxidation and subsequently reduce H^+ production. This is because a lower level of fatty acid oxidation can reduce the ratios of acetyl CoA/CoA and NADH/NAD$^+$, and reduce the inhibition of the rate-limiting enzyme for glucose oxidation, PDH. Another approach to decrease the detrimental effects of fatty acids is to stimulate glucose oxidation by direct activation of PDH. Stimulation of glucose oxidation will improve the coupling of glycolysis to glucose oxidation and thereby reduce H^+ production[72,78]. Dichloroacetate (DCA) is a stimulator of glucose oxidation, which activates PDH by inhibition of PDH kinase[150]. Previous studies have shown that DCA increases glucose oxidation, decreases H^+ production from glucose metabolism and improves cardiac function and efficiency during reperfusion of ischemic hearts[72,78]. However, whether these changes in H^+ production translate into changes in pH_i has not been determined. Small clinical trials have shown that DCA has beneficial effects in the treatment of angina[151] and congestive heart failure[152]. Stimulation of glucose oxidation by ranolazine and trimetazidine can inhibit fatty acid oxidation and both of these agents have cardioprotective properties (see 153 for review). L-carnitine can also stimulate glucose oxidation by decreasing intramitochondrial acetyl-CoA/CoA ratio[153]. Worthy of mention, hyperthyroidism is closely associated with a high level of energy metabolism, especially glucose utilization[154]. Although the effects of thyroid hormone are thought to be due to changes in myocardial gene expression, attention has recently focused on acute actions of 3,5,3'-triiodo-L-thyronine (T_3). A number of basic and clinical studies have shown that treatment with supraphysiological concentrations of T_3 has cardioprotective effects in postischemic hearts (see 155 for review). It is possible that the beneficial effects of T_3 on the recovery of cardiac mechanical function is due to its activation of glucose oxidation with an associated reduction of H^+ production. In the present studies, the effects of T_3 on glucose metabolism, H^+ production and the recovery of cardiac function and efficiency in postischemic hearts were investigated.

Improving coupling between glycolysis and glucose oxidation by inhibiting excessive rates of glycolysis is another approach to decrease H^+ production. Previous studies showed that adenosine inhibits glycolysis and improves the coupling of glycolysis to glucose oxidation, decreases proton production from the hydrolysis of glycolytically derived ATP, and improves cardiac efficiency during reperfusion[156,157]. In the present studies, the effects of inhibition of glycolysis on intracellular H^+ and pH_i were also investigated. It is well known that glycolysis is inhibited by H_2O_2 generated free radicals[158-160]. This means that H^+ production may be reduced. However, recent studies have shown that intracellular acidosis develops in cultured endothelial cells and myocytes subjected to H_2O_2 overload[158,159]. It is widely accepted that H_2O_2 depletes intracellular high energy phosphate contents. Since the activity of NHE1 and vacuolar-H^+ ATPase both rely on ATP[111,112,143], it is estimated that the above H^+ extrusion pathways are blocked in H_2O_2-treated cells and this may be the major reason responsible for intracellular acidosis in H_2O_2-treated hearts. However, in cultured cells, glucose metabolism is very low and glucose is often the only energy substrate available to the cells [158,159] which, of course, does not reflect the status of normal energy metabolism. Furthermore, energy metabolism is closely associated with workload. We therefore re-investigated the effects of H_2O_2 on pH_i and intracellular H^+ production in isolated working hearts under more physiological conditions of energy supply and demand.

Experimental approach

An isolated working heart model, perfused with both fatty acids and glucose was applied in the present studies. This is because fatty acids are the major fuel for the heart and in this model, the heart was subjected to physiological workloads. Therefore, the status of normal energy metabolism is well reflected. ^{31}P-NMR was also used since it is the gold standard for the measurement of pH_i. The advantage of this technique, compared with pH-sensitive fluorescent dyes (SNARF or BCECF), which are widely applied in isolated cells, is that it is non-invasive and can be applied to pH studies on whole ischemic and postischemic hearts. The sensitivity of the method is also high enough to resolve time-courses as rapid as a few minutes. Meanwhile the dynamic change of energy phosphate contents can also be monitored. This is a tremendous help and provides a time course of metabolic events. In the present studies, an isolated working heart model in which both energy metabolism and ^{31}P-NMR measurement could be made was developed (see Chapter 2 for details). This allowed us to answer the question: will altering the source of protons change pH_i in isolated working rat hearts during reperfusion?

Optimizing energy substrate preference by the heart during reperfusion is an exciting approach to treating ischemic heart disease. However, the relationship between glucose metabolism and alterations in H^+ production and clearance during ischemia and reperfusion is poorly understood. Direct evidence that decreasing H^+ production by improving the coupling of glucose metabolism can accelerate the recovery of pH_i during reperfusion is lacking. Therefore the focus of this thesis was to address the relationship between a decrease in H^+ production by pharmacologically improving the coupling of glycolysis to glucose oxidation (stimulation of glucose oxidation, inhibition of glycolysis) and pH_i, cardiac mechanical function and efficiency in postischemic hearts. Since inhibition of fatty acid oxidation can also stimulate glucose oxidation and improve the coupling of glucose metabolism (see 153 for review), the effects of down-regulation of fatty acid oxidation on pH_i, cardiac function and efficiency were also investigated.

Hypothesis

Both the source and fate of protons influence the recovery of mechanical function and cardiac efficiency in the reperfused postischemic heart. Inhibition of the source of H^+ during reperfusion by improving the coupling between glycolysis and glucose oxidation will increase the rate of recovery of pH_i and improve recovery of mechanical function and cardiac efficiency in the reperfused ischemic heart. Inhibition of NHE1 will improve the recovery of cardiac contractile function and efficiency during reperfusion, despite a slower recovery of pH_i.

Specific objectives

1. To develop an isolated working heart model to measure pH_i by using ^{31}P-NMR technique. This model needs to meet the requirements for measuring both cardiac energy metabolism (fatty acid and glucose metabolism) and pH_i (see Chapter 2 for details).
2. To determine the importance of glucose metabolism as a source of H^+ by measuring the consequences of T_3 and DCA on rates of glycolysis, glucose oxidation, H^+ production, recovery of pH_i, mechanical function and cardiac efficiency.
3. To determine the effects of inhibition of NHE1 on the fate of H^+, as determined by the recovery of pH_i, as well as cardiac function and efficiency in postischemic hearts perfused with a high level of fatty acid, which facilitates H^+ production by inhibiting glucose oxidation.

4. To evaluate the consequences of combined alteration of both the source and fate of H^+ on the recovery of pH_i, mechanical function and cardiac efficiency.
5. To determine the effects of inhibition of glycolysis by H_2O_2 on H^+ production, pH_i, cardiac function and efficiency in aerobic hearts perfused with a high level of fatty acid.

Figure 1-1. Overall pathways of myocardial energy substrate metabolism (see text for details).

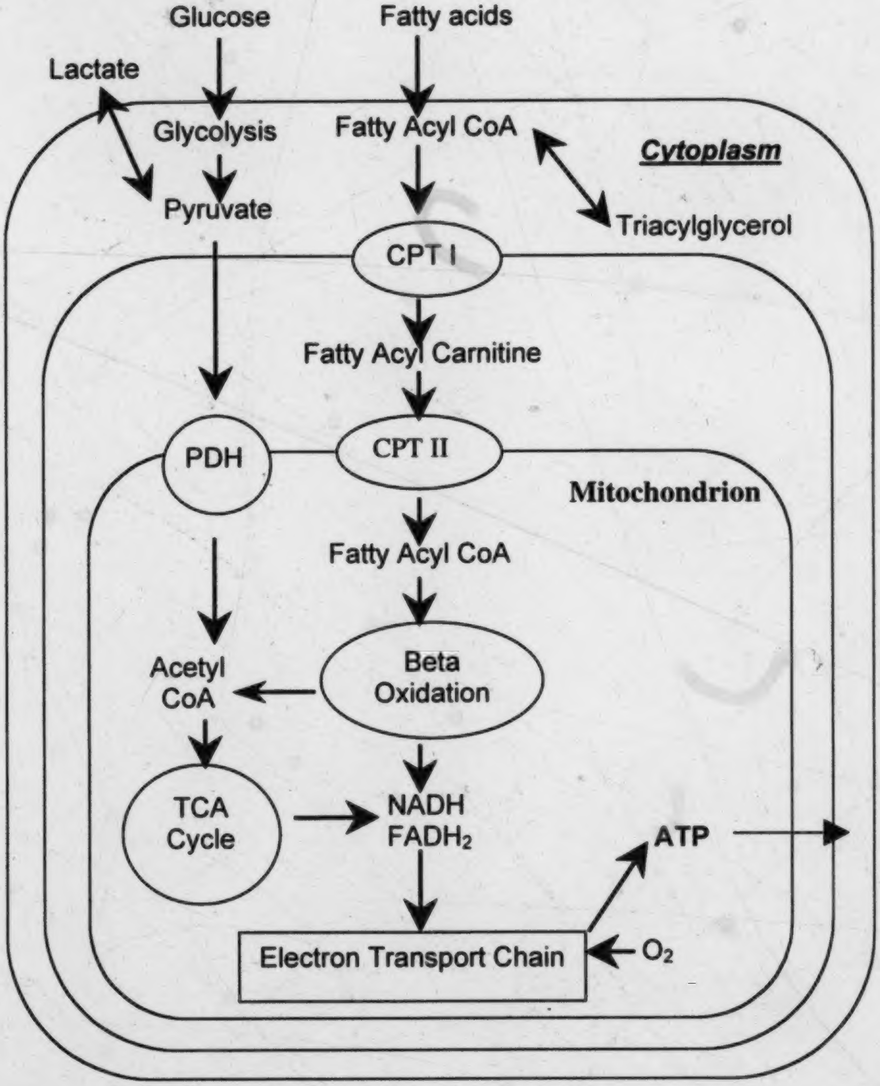
Glucose
Fatty acids
Lactate
Glycolysis
Fatty Acyl CoA
Cytoplasm
Pyruvate
Triacylglycerol
CPT I
Fatty Acyl Carnitine
PDH
CPT II
Mitochondrion
Fatty Acyl CoA
Acetyl CoA
Beta Oxidation
TCA Cycle
NADH
FADH2
ATP
Electron Transport Chain
O2

Figure 1-2. Pathways of glucose metabolism and generation of H^+.

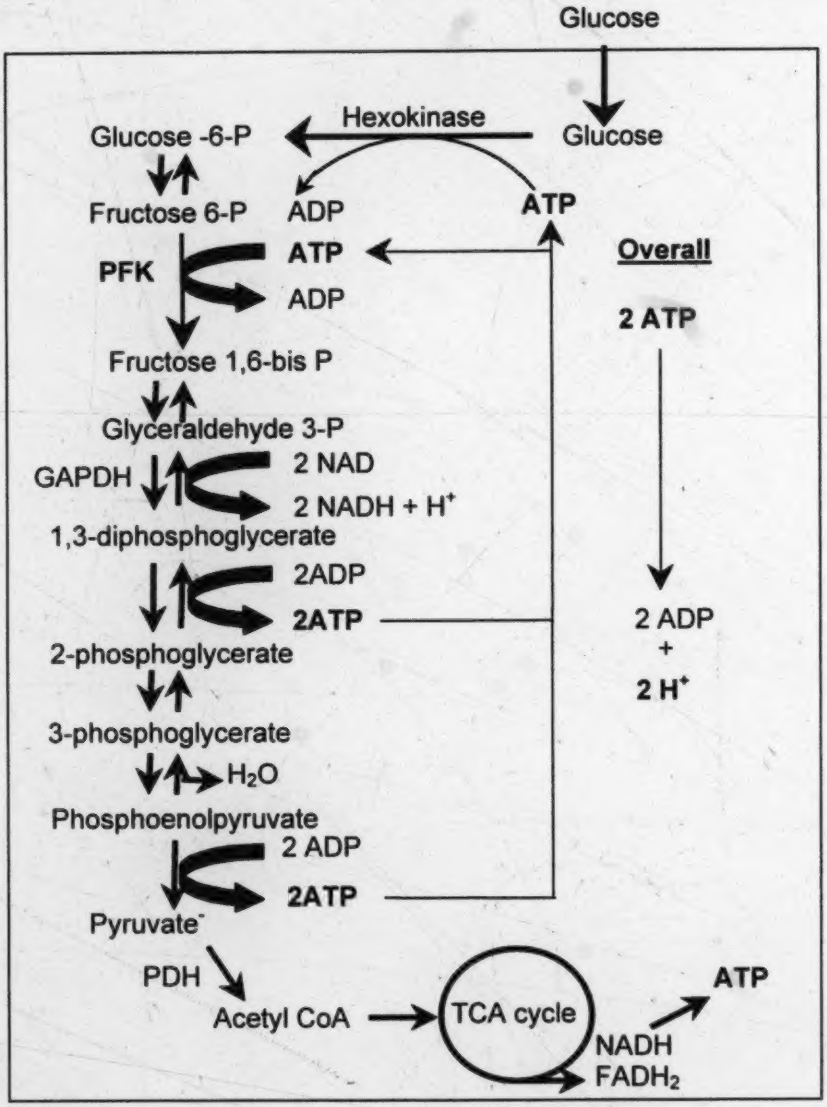
Glucose
Hexokinase
Glucose -6-P
Glucose
ADP
ATP
Fructose 6-P
PFK
ATP
ADP
Overall
2 ATP
Fructose 1,6-bis P
Glyceraldehyde 3-P
GAPDH
2 NAD
2 NADH + H⁺
1,3-diphosphoglycerate
2ADP
2ATP
2 ADP
+
2 H⁺
2-phosphoglycerate
3-phosphoglycerate
H₂O
Phosphoenolpyruvate
2 ADP
2ATP
Pyruvate⁻
PDH
Acetyl CoA
TCA cycle
ATP
NADH
FADH₂

Figure 1-3. Regulation of the activity of pyruvate dehydrogenase (PDH).

PDH is inactivated by phosphorylation which is catalysed by PDH kinase (PDHK). PDHK is activated by increases in the concentration ratios of acetyl-CoA/CoA and $NADH/NAD^+$ and inhibited by dichloroacetate (DCA) or by pyruvate from glycolysis. Dephosphorylation of phosphorylated PDH (PDH-P) by PDH phosphatase which is activated by Ca^{2+} can activate PDH.

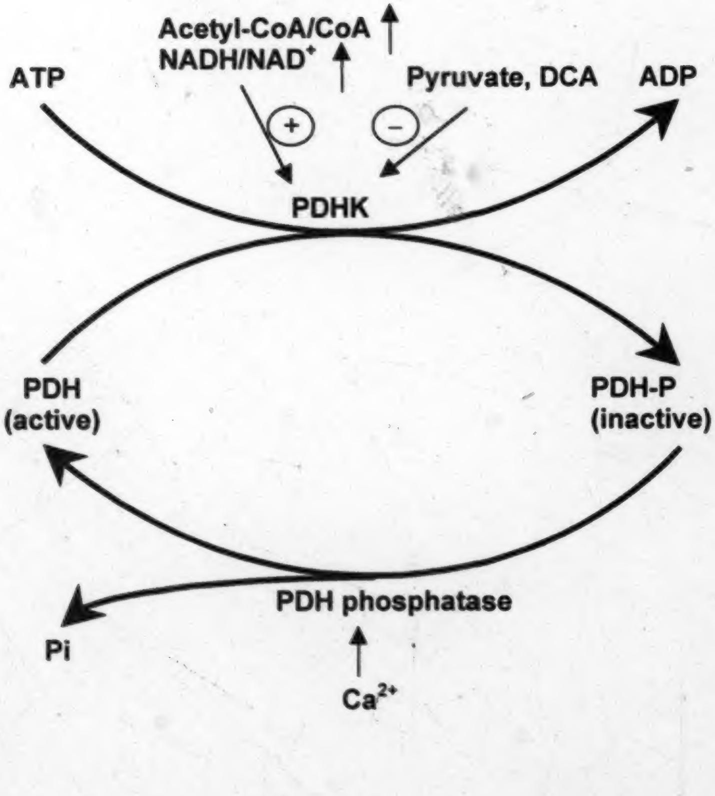
Acetyl-CoA/CoA ↑
NADH/NAD+ ↑
ATP
Pyruvate, DCA
ADP
+
−
PDHK
PDH
(active)
PDH-P
(inactive)
PDH phosphatase
Pi
Ca2+

Figure 1-4. Essential versus nonessential fuels.

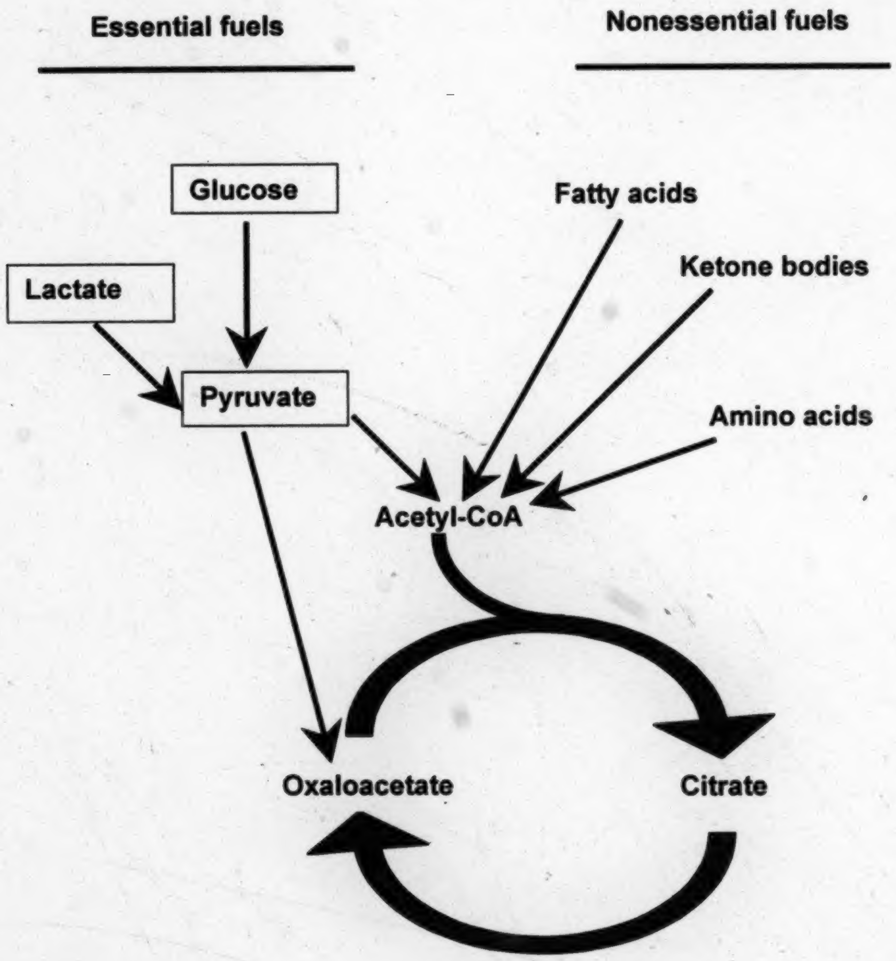
Essential fuels
Nonessential fuels
Glucose
Lactate
Pyruvate
Fatty acids
Ketone bodies
Amino acids
Acetyl-CoA
Oxaloacetate
Citrate

Figure 1-5. Putative topological model of 815–amino acid NHE1 showing 12 transmembrane-spanning segments and hydrophilic carboxyl terminus, with indications of proposed regulatory sites[107]. Localization of the putative H^+ sensor that accounts for the sensitivity of NHE1 to pH_i has not been confirmed but likely resides in the lipophilic transmembrane region.

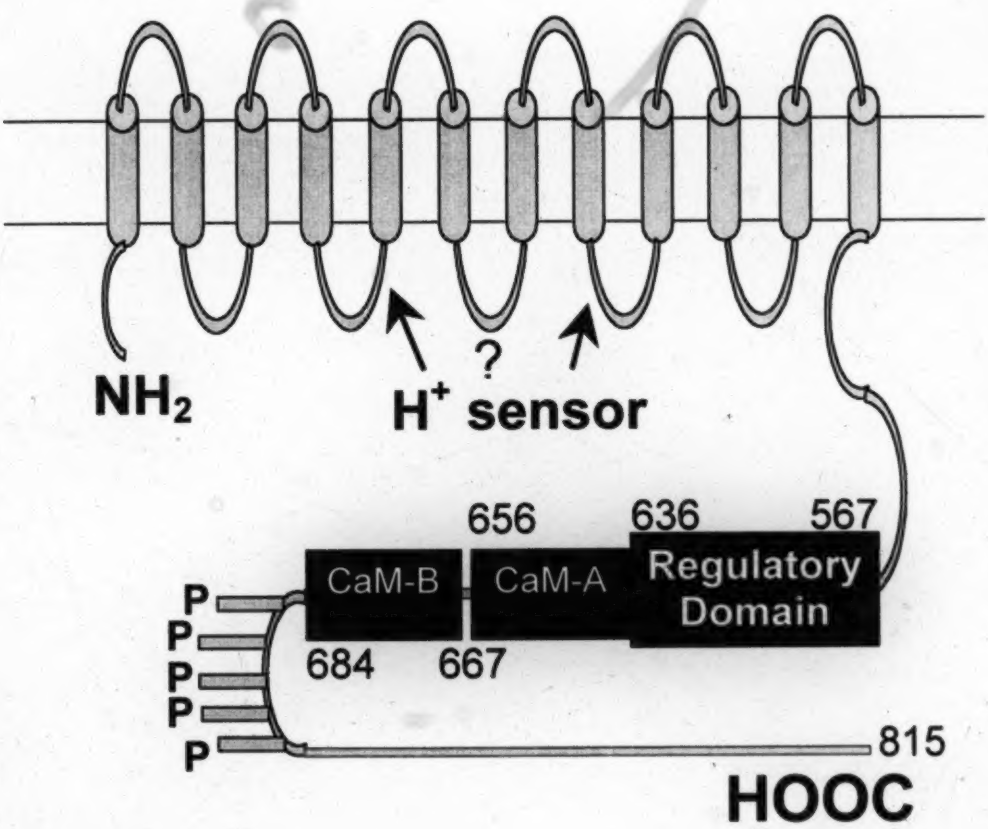
NH₂
?
H⁺ sensor
656
636
567
CaM-B
CaM-A
Regulatory Domain
684
667
P
P
P
P
P
815
HOOC

Table 1-1. Beneficial effects of NHE1 inhibitors in the ischemic myocardium

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Improved recovery of left ventricular function after ischemia[82,84]

Reduction in infarct size and necrosis[84]

Reduced ischemia- and reperfusion-associated contracture[82]

Attenuation of ion dyshomeostasis during ischemia and reperfusion

Reduced apoptosis[84]

Reduction in ischemia- and reperfusion-induced arrhythmias[89]

Preservation of high-energy phosphates [82]

Reduced postinfarction mortality[84]

Reduced mortality and incidence of myocardial infarction after coronary bypass surgery*[107]

Improved left ventricular function after balloon angioplasty*[107]

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*Clinical study.

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Chapter 2

General experimental methods

1. Materials

D-[5-^{3}H]glucose, D-[U-^{14}C]glucose, [1-^{14}C]palmitate were purchased from Du Pont-New England Nuclear. Bovine serum albumin (fraction V) was obtained from Roche. Hyamine hydroxide (methylbenzethonium; 1 M in methanol solution) was obtained from ICN Radiochemicals. T_3 was obtained from Sigma Chemical Company. DCA was obtained from BDH Chemical Ltd. Cariporide was provided by Hoechst Marion Roussel company. Dowex 1X4 anion exchange resin (200-400 mesh chloride form), Dowex AG50 WX8 (200-400 mesh, hydrogen form), and Dowex AG1X8 (200-400 mesh, acetate form), were obtained from Bio-Rad. ACS counting scintillant was purchased from Amersham Canada Ltd. Ecolite counting scintillant was obtained from ICN Biomedicals Canada Ltd. Acetyl CoA was obtained from Boehringer-Mannheim. The AMARA peptide used in the AMPK assay was synthesized by the Alberta Peptide Institute. [^{32}P]ATP and [^{14}C]sodium bicarbonate were obtained from ICN radiopharmaceuticals. All other chemicals were reagent grade.

2. Isolated working heart perfusions

All animal protocols, housing, surgical manipulations, and euthanization were approved by the Health Sciences Animal Welfare Committee at the University of Alberta, and conform to the standards set by the Canadian Council of Animal Care. Rat hearts were quickly cannulated for isolated working heart perfusions as described previously[1-3]. In brief, male Sprague-Dawley rats (Charles River Laboratories, 0.30-0.35 kg) were anesthetized with pentobarbital sodium (60 mg • kg^{-1} IP) and hearts were quickly excised. The aorta of each heart was cannulated within 30 sec of removal from the rat and a retrograde perfusion at 37°C was initiated at a hydrostatic pressure of 60 mm Hg. Hearts were trimmed of excess tissue, and the pulmonary artery and the opening to the left atrium were then cannulated. After 15 min of Langendorff perfusion, hearts were switched to the working mode by clamping the aortic inflow line from the Langendorff reservoir and opening the left atrial inflow line. The perfusate was delivered from an oxygenator into the left atrium at a constant preload pressure of 11.5 mm Hg. Perfusate was ejected from spontaneously beating hearts into a compliance chamber (containing 1 ml of air) and into the aortic outflow line. The afterload was set at a hydrostatic pressure of 80 mm Hg. The perfusate volume was 100 ml.

Spontaneously beating hearts were used in all studies. Heart rate and peak systolic pressure (PSP) were measured with a Gould P21 pressure transducer connected to the aortic outflow line. Developed aortic pressure was calculated as PSP minus diastolic pressure. Coronary vascular resistance (CVR, mmHg • min • ml^{-1}) was calculated as mean aortic pressure divided by coronary flow. Cardiac output and aortic flow were measured with Transonic T206 ultrasonic 2N probes in the preload and afterload lines, respectively. Coronary flow was calculated as the difference between cardiac output and aortic flow.

The O_2 contents of the perfusate were measured using YSI micro oxygen electrodes placed in the preload line and in a line originating from the cannulated pulmonary artery. Myocardial O_2 consumption (MVO_2) was calculated according to the Fick principle, using coronary flow rates and the arteriovenous difference in perfusate O_2 concentration. Cardiac work was calculated as the product of PSP and cardiac output. Cardiac efficiency was defined as a ratio of cardiac work to MVO_2. All working hearts were perfused with Krebs-Henseleit solution containing 2.5 mM Ca^{2+}, 5.5 mM glucose, 3% bovine serum albumin (fraction V, Boehringer Mannheim), and 0.4 or 1.2 mM palmitate. Palmitate was prebound to the albumin as described previously[4]. The perfusate was recirculated and continuously gassed with 95% O_2 and 5% CO_2, which provided pH around 7.35-7.45.

At the end of each perfusion protocol, hearts were quickly frozen with Wollenberger clamps cooled to the temperature of liquid N_2. The atrial tissue was dried in an oven for 12 hours at 100 °C and weighed. The frozen ventricular tissue was weighed and powdered in a mortar and pestle cooled to the temperature of liquid N_2. A portion of the powered tissue was used to determine the dry weight-to-wet weight ratio. The dried atrial weight, frozen ventricular weight, and ventricular dry weight-to-wet weight ratio were then used to determine the total dry weight of the heart.

3. Determination of pH_i by ^{31}P-NMR spectroscopy

3.1 Isolated working heart model for ^{31}P-NMR and energy metabolism measurements (for Chapters 4-6).

Isolated working heart models adapted for use within the confines of a magnet for NMR studies have been described previously[4,5]. In the present studies, measurement of glucose metabolism using radiolabeled tracers required that the entire perfusion system be sealed[6]. The author developed a perfusion system that permitted efficient operation of a closed system within the confines of a high field magnet. The detailed tubing connection for the working heart has been shown previously[4,5]. The cannula assembly for the working heart is illustrated in Fig. 2-1. This structure supports six perfusion lines: 1) a vacuum line to remove coronary effluent around the heart that was positioned so that the heart was always immersed by perfusate up to the level of the aorta, 2) an aortic outflow line, 3) a pressure transducer line that connects with the aortic outflow line close to the heart (not shown), 4) a vacuum line to remove extra perfusate from the oxygenator so that preload was kept at 11.5 mmHg, 5) a left atrial inflow line (for working heart perfusions), 6) a collection line for gas containing $^{14}CO_2$ that exited the oxygenator. The tubing connecting the cannula assembly and perfusion reservoirs comprise an approximately 104 cm "umbilical" that allows the cannula assembly to be inserted in the NMR magnet (clear bore of 10 cm) while the perfusion apparatus itself remains outside of the bore of the magnet. The bottom part of the cannula assembly (heart chamber) was designed to fit within a 25-mm NMR tube. In addition, the aortic outflow line, after leaving the compliance chamber, proceeded out of the magnet and into the perfusate reservoir for recirculation. This aortic outflow line provided the resistance necessary to generate an 80 mm Hg hydrostatic pressure (afterload) on the heart. Perfusion lines were water-jacked to maintain perfusion temperatures of 37 °C. Total perfusate volume was 100 ml.

Perfusion of hearts within the NMR magnet was identical to the normal working heart perfusions (see previous section). After conversion to the working mode the heart was cautiously inserted into the NMR tube (outer diameter 25 mm). In general, arrhythmia, fluctuation and reduction of the aortic flow occurred during this experimental procedure, but disappeared quickly after hearts were adjusted within the magnet. It is noteworthy that one of the major problems described in previous studies of isolated working hearts designed for ^{31}P-NMR[4,5] was that the long tube connecting the heart within the magnet bore to the perfusion apparatus required a higher pressure in the hearts to overcome inertia and friction. This resulted in a dramatic depression of cardiac function compared with that seen in normal working hearts. To avoid this limitation, an oxygenator, which also functioned as a heat exchanger, was inserted into the magnet close to the heart chamber. Furthermore, the length of the oxygenator was increased to 37 cm (3.2 cm outer diameter), which efficiently increased the surface area in contact with carbogen. In order to diminish the resistance of perfusion system, the inner diameters of the heart cannulae were enlarged (2.5 mm for aortic cannula and 3.0 mm for left atrial cannula). All working hearts were perfused with Krebs-Henseleit solution (same as normal condition) except that 1.2 mM KH_2PO_4 was replaced by 1.2 mM KCl (there was no change in cardiac function).

The effluents from the two vacuum lines (coronary effluent and oxygenator outflow) and the aortic outflow line were pumped into the perfusate reservoir and this created a positive pressure. In contrast, there was a negative pressure inside the heart chamber due to the perfusate that was removed. To normalize the pressure inside the heart chamber, another gas (95% O_2 and 5% CO_2) line was inserted into the chamber. This normalization of chamber pressure allowed the flow through the aortic cannula (without cannulated heart) to reach its normal range (90 to 100 ml • min^{-1}). To normalize the pressure inside the perfusate reservoir, another $^{14}CO_2$ collection line was inserted into the hyamine trap (see following metabolism section) and connected to the reservoir.

Previously, several problems remained after modification of the heart perfusion system that led to depressed cardiac function. In order to ensure magnetic field homogeneity, the heart must work while immersed within the perfusate. This causes an additional pressure on the heart that is not present under normal working heart conditions. This problem was solved by normalization of the pressure inside the heart chamber. A second problem was that the confines of the NMR tube impaired the movement of the heart during contraction and relaxation. This problem was solved by using a larger diameter NMR tube (25 mm), which did not significantly increase the signal to noise ratio (see Fig. 2-2 for spectra). With these modifications, there were no significant differences in cardiac function between hearts perfused under normal conditions and hearts perfused under the modified condition for ^{31}P-NMR measurements (Table 2-1).

3.2 Experimental conditions for ^{31}P-NMR study*

^{31}P-NMR spectra were acquired using a Bruker Advance 500 spectrometer in conjunction with a 120 mm vertical bore 11.7 T magnet (Magnex, Oxford, U.K.). The working hearts were positioned within a 25 mm dual channel NMR probe (Morris Instruments). Field homogeneity was adjusted by shimming the proton signal using the ^{1}H channel and yielded line widths of approximately 0.1 ppm. ^{31}P-NMR spectra were acquired at 202.4 MHz with a time resolution of 2.25 min (1.12 min at the end of ischemia and first 10 min of reperfusion), using a 60 °C pulse, and a 1.8 sec recycle time. Spectra were processed using WINNMR (Bruker) by summing 72 (or 36) free induction decays and subjected to Fourier Transformation following exponential multiplication (Line Broadening=30). The content of high energy phosphates was determined by intergration of the areas under the peaks. During an initial stabilization period of 30 min of aerobic perfusion, which included the time required for tuning of the probe and shimming of the magnet, baseline spectra were required before the onset of ischemia. pH_i

was determined from the chemical shift of Pi relative to PCr with a calibration curve obtained by titrating Pi in a solution mimicking the intracellular milieu[8]. The rate of recovery of pH_i was defined as a ratio of the change in pH_i to the corresponding time period (until pH_i recovered to preischemic level) after ischemia and expressed as pH units • min^{-1}.

* ^{31}P-NMR analysis was performed in collaboration with Dr. John C. Docherty, National Research Council, Winnipeg.

4. Measurement of glycolysis, glucose oxidation, and palmitate oxidation

Glycolysis and glucose oxidation rates were measured simultaneously by perfusing hearts with [5-^{3}H/U-^{14}C]glucose[6,7]. Rates of glycolysis were measured by quantitative determination of the amount of ^3H_2O liberated from the labeled [5-^{3}H]glucose at the enolase step of glycolysis[6,7]. ^3H_2O was separated from [^{3}H]glucose and [^{14}C]glucose (used to measure glucose oxidation, see following section) using chromatography columns containing Dowex 1x4 anion exchange resin (200-400 mesh) suspended in 0.4 M potassium tetraborate. The Dowex resin in the columns (resin volume of 0.4 ml) was extensively washed with H_2O prior to use. A 0.2 ml volume of perfusate was added to the column and eluted into scintillation vials with 0.8 ml of H_2O. Following addition of Ecolite scintillant, the samples were subjected to standard double isotope counting procedures to detect ^3H_2O and residual [^{3}H/^{14}C]glucose. The Dowex columns were found to retain 98-99.6% of the total [^{3}H]glucose and [^{14}C]glucose present in the perfusate. The ^3H_2O was corrected for the small amount of [^{3}H]glucose that passed through the column. This could be accomplished since an equal amount of [^{14}C]glucose also passed through the column and could be used as an internal standard for the degree of [^{3}H]glucose contamination in the ^3H_2O sample. Correction was also made for the degree of spillover from [^{14}C] into the [^{3}H] counting window by measuring this degree of spillover using standards containing only [^{14}C]glucose. Glycolytic rates are expressed as nmol of glucose metabolized • min^{-1} • g dry wt^{-1}.

Rates of glucose oxidation and fatty acid oxidation were measured in separate groups of hearts by quantitatively collecting $^{14}CO_2$ produced by the heart from [U-^{14}C]glucose or [1-^{14}C]palmitate in the perfusate, as described previously[6,7]. $^{14}CO_2$ production included $^{14}CO_2$ released as a gas in the oxygenation chamber and $^{14}CO_2$ dissolved as $H^{14}CO_3^-$ in perfusate. The gaseous $^{14}CO_2$ was trapped in hyamine hydroxide solution through the exhaust line in the perfusion system (see Fig. 2-1). The dissolved $^{14}CO_2$ as $H^{14}CO_3^-$ was released and trapped on filter paper saturated with hyamine hydroxide in the central well of 25 ml stoppered flasks after perfusate samples were acidified by the addition of 1 ml of 9N H_2SO_4. Rates of glucose or fatty acid oxidation are expressed as nmol of glucose or palmitate metabolized • min^{-1} • g dry wt^{-1}.

5. Calculation of H^+ production from glucose utilization

If glucose passes through glycolysis to lactate and the ATP so formed is hydrolyzed, a net production of 2 H^+ per molecule of glucose occurs[7,9]. In contrast, if glycolysis is coupled to glucose oxidation, the net production of H^+ is zero. Therefore, the overall rate of H^+ production derived from glucose utilization was determined by subtracting the rate of glucose oxidation from the rate of glycolysis and multiplying by 2.

6. Calculation of tricarboxylic acid cycle (TCA) acetyl-CoA production

The rate of acetyl-CoA production for the TCA cycle was calculated based on 2 acetyl-CoA being produced from each molecule of glucose oxidized and 8 acetyl-CoA from each molecule of palmitate oxidized[9,10].

7. 5'-AMP-activated protein kinase (AMPK) and acetyl-CoA carboxylase (ACC) assays

7.1 Extraction of AMPK and ACC

Approximately 200 mg of frozen tissue was homogenized in a buffer containing Tris • HCl (50 mM, pH 7.5 at 4°C), mannitol (0.25 M), NaF (50 mM), sodium pyrophosphate (5 mM), EDTA (1 mM), EGTA (1 mM), dithiothreitol (1 mM), and the following protease inhibitors: phenylmethylsulfonyl fluoride (1 mM), soybean trypsin inhibitor (4 µg • ml^{-1}), and benzamidine (1 mM). Samples were centrifuged at 14,000 g for 20 min at 4°C. The supernatant was brought to 2.5% (w/V) polyethylene glycol (PEG) with 25% (wt • vol^{-1}) PEG 6000 and was agitated for 10 min at 4°C. Samples were then spun at 10,000 g for 10 min at 4°C. The supernatant was then made up to 6% PEG 6000 (W/V) using a PEG 6000 stock and was stirred once again for 10 min at 4°C. This fraction was spun at 10,000 g for 10 min, and the precipitate was washed with homogenization buffer containing 6% PEG 6000 (W/V). This was followed by a final centrifugation at 10,000 g, after which the protein concentration in the supernatant was measured using a Sigma bicinchoninic acid protein kit.

7.2 ACC assay

ACC activity in the PEG 6000 fractions was measured using a CO_2 fixation method[11]. Briefly, 5 µl of the PEG fraction, containing 20 µg of total protein, were added to a reaction mixture (final volume, 165 µl) containing Tris • acetate (60.6 mM), BSA (1 mg • ml^{-1}), 2-mercaptoethanol (1.32 µM), ATP (2.21 mM), acetyl-CoA (1.06 mM), magnesium acetate (5.0 mM), and $NaHCO_3$ (18.08 mM), pH 7.4. Samples were incubated at 37°C for 10 min, and the reaction was stopped by adding 25 µl of 10% perchloric acid. Samples were then spun for 20 min at 3,500 g, and 160 µl of supernatant were placed in minivials and dried in a fume hood overnight. H_2O (100 µl), followed by scintillant, was added to the vials, and the vials were counted. ACC activity was expressed as the amount of malonyl-CoA produced • min^{-1} • mg $protein^{-1}$.

7.3 AMPK assay

AMPK activity was measured by following the incorporation of ^{32}P into a synthetic peptide[11]. Briefly, 2 µl of the PEG fraction were added to a reaction mixture (final volume, 25 µl) composed of HEPES-NaOH (40 mM), NaCl (80 mM), glycerol (8%, wt • vol^{-1}), EDTA (0.8 mM), AMARAASAAALARRR (AMARA) peptide (200 µm), dithiothreitol (0.8 mM), [γ-^{32}P]ATP (200 µM), $MgCl_2$ (5 mM), and 0.18% Triton X-100. Samples were also incubated in the presence or absence of 200 µM AMP. This mixture was incubated for 3 min at 30°C. From this incubation mixture, 15 µl were spotted on 1-cm^2 phosphocellulose paper. The paper was then washed four times for 10 min each with 150 mM phosphoric acid, followed by a 5-min acetone wash. The papers were then dried and counted for radioactivity. AMPK activity was expressed as pmol ^{32}P incorporated into the AMARA peptide • min^{-1} • mg $protein^{-1}$.

8. Malonyl-CoA decarboxylase (MCD) assay*

MCD activity was measured according to a protocol described by Dyck et al[12]. The product of MCD, acetyl-CoA, was detected[12,13]. Acetyl-CoA derived from MCD was incubated in the presence of [^{14}C]oxaloacetate and citrate synthase (0.73 µU • μl^{-1}) to form citrate. The [^{14}C]oxaloacetate was initially produced by a 20-min transamination reaction performed at room temperature utilizing L-[U-^{14}C]aspartate (2.5 µCi • ml^{-1}) and 2-oxoglutarate (2 mM)[12,13]. One of the advantages of this assay was that extensive purification of MCD was not required before assay.

To initiate the MCD assay, heart homogenates were incubated in a 210 µl reaction mixture (0.1 M Tris, pH 8, 0.5 mM dithiothreitol, 1 mM malonyl-CoA) for 10 min at 37°C, in the presence or absence of NaF (50 mM) and NaPP$_i$ (5 mM)[12]. The reaction was stopped by the addition of 40 µl of perchloric acid (0.5 mM), neutralized with 10 µl of 2.2 M $KHCO_3$ (pH 10) and centrifuged at 10,000 g for 5 min to remove precipitated proteins. The incubation of the heart sample with malonyl-CoA allowed for the conversion of malonyl-CoA to acetyl-CoA, which was then combined with [^{14}C]oxaloacetate (0.17 µCi • ml^{-1}) to produce [^{14}C]citrate. All reactions were carried out in the presence of *N*-ethylmaleimide, which removes excess CoA remaining in the latter stages of the reaction so that the citrate present cannot generate non-MCD-derived acetyl-CoA. Unreacted [^{14}C]oxaloacetate was removed from the reaction mixture by the addition of sodium glutamate (6.8 mM) and aspartate aminotransferase (0.533 µU • µl^{-1}), followed by a 20-min incubation at room temperature. This allowed for transamination of unreacted [^{14}C]oxaloacetate back to [^{14}C]aspartate. The resulting solution was then stirred in a 1:2 suspension of Dowex AG 50W-8X resin (100-200 mesh) and centrifuged at 400 g for 10 min. The pelleted Dowex fraction removed [^{14}C]aspartate, whereas the supernatant contained [^{14}C]citrate.

The supernatant fraction was then counted for ^{14}C present in the form of $[^{14}C]$citrate. The amount of acetyl-CoA produced by MCD was then quantified by comparison to acetyl-CoA standard curves that had been subjected to the identical assay conditions as described above. MCD activity was quantified by comparison with acetyl-CoA standards.

* MCD assay was performed by Miss Jennifer Watson

9. PDH assay

Frozen ventricular tissue was weighed and powered with a mortar and pestle cooled in liquid nitrogen. The PDH analysis was performed using a modification of the radioisotopic-coupled enzyme assay developed by Constantin-Teodosiu et al.[14] Frozen ventricular tissue samples were homogenized for 30 sec in the presence of 370 µl of 200 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, 5 mM EGTA, 50 mM Tris-HCl, 50 mM NaF, 5 mM DCA, and 0.1% Triton X-100 (V/V) (pH 7.8). To measure total PDH activity, NaF was omitted from the homogenization buffer, and 10 mM glucose and 2.25 U hexokinase • ml^{-1} were added to the buffer. The homogenate was then incubated for 15 min at 37 °C in the presence of 200 mM sucrose, 4.8 mM $CaCl_2$, 25 mM DCA, 50 mM KCl, 0.5 mM EGTA, 100 mM glucose, 2.25 U hexokinase, and 50 mM Tris-HCl, pH 7.4. The PDH assay for both active (PDHa) and total PDH (PDHt) was initiated by addition of 100 µl of homogenate to 480 µl of assay buffer containing (in mM) 150 Tris-HCl,0.75 NAD^+, 0.75 CoA, and 1.5 thiamine pyrophosphate, pH 7.8. The reaction was initiated by addition of pyruvate (30 µl of a 26 mM stock solution) to a final concentration of 1 mM. After 10 min, the reaction was terminated by the addition of 210 µl of 0.5 M perchloric acid. The solution was then neutralized and centrifuged, and the supernatant acetyl-CoA content was measured. Activity was based on the measurement of acetyl-CoA in which acetyl-CoA formed from the PDH assay (active or total) was converted to [^{14}C]citrate in the presence of citrate synthase and [^{14}C]aspartate. Sodium glutamate and aspartate aminotransferase were used to remove excess [^{14}C]oxaloacetate after the citrate synthase reaction by transamination of unreacted oxaloacetate to [^{14}C]aspartate from [^{14}C]citrate. Dowex (50W-X8, 100-200 mesh) was then used to separate [^{14}C]aspartate from [^{14}C]citrate. Acetyl-CoA content of supernatant samples was quantified by comparison with acetyl-CoA standard curves run in each experiment.

10. Determination of CoA esters*

Detection and quantification were performed by extracting CoA esters from powdered tissue into 6% perchloric acid (V/V) and measuring with a modified HPLC procedure[15]. Essentially, each sample (100 µl) was run through a precolumn cartridge (C_{18}, size 3 cm, 7 µm) and a Microsorb Short-one column (type C_{18}, particle size 3 µm, size 4.6 × 100 mm) on a Beckman System Gold HPLC. Absorbance was set at 254 nm and flow rate at 1 ml • min^{-1}. A gradient was initiated using buffer A (0.2 M NaH_2PO_4, pH 5.0) and buffer B (0.25 M NaH_2PO_4 and acetonitrile, pH 5.0) in a ratio of 80:20 (vol • vol^{-1}). Conditions were maintained at initial levels for 2.5 min (97% buffer A and 3% buffer B) and were changed thereafter to 18% buffer B over 5 min using Beckman's curve no. 3. The gradient was changed linearly at 15 min to 37% buffer B over 3 min and subsequently to 90% buffer B over 17 min. At 42 min the composition was returned linearly back to 3% buffer B over 0.5 min, and at 50 min column equilibration was complete. Peaks were integrated by the Beckman System Gold software package.

* HPLC analysis was performed by Mr. Ken Strynadka.

11. Statistical analysis

All data are presented as the mean ± S.E.M. The data were analyzed with the statistical program Instat 2.01. Statistical comparisons between groups were carried out by ordinary or repeated measures ANOVA with post hoc Bonferroni test for multiple comparisons. When the data sets were unevenly distributed, the Kruskal-Wallis nonparametric test were used to determine the significance of differences. Two-tailed values of $p<0.05$ were considered significant.

Figure 2-1. Isolated working rat heart model for ^{31}P-NMR and energy metabolism studies.

1.Vacuum line; 2. Plexiglass block; 3. Aortic outflow line; 4. Vacuum line; 5. Left atrial line; 6. $^{14}CO_2$ collection line (from oxygenator); 7. Pulmonary arterial cannula to measure MVO_2; 8. Left atrial cannula; 9. Heart chamber; 10. Aortic cannula; 11.Plastic support dowel; 12. Oxygenator (95% O_2, 5% CO_2);13. Perfusate reservoir; 14. Peristaltic pump; 15. $^{14}CO_2$ collection line (from perfusate reservoir);16. Left atrial line.

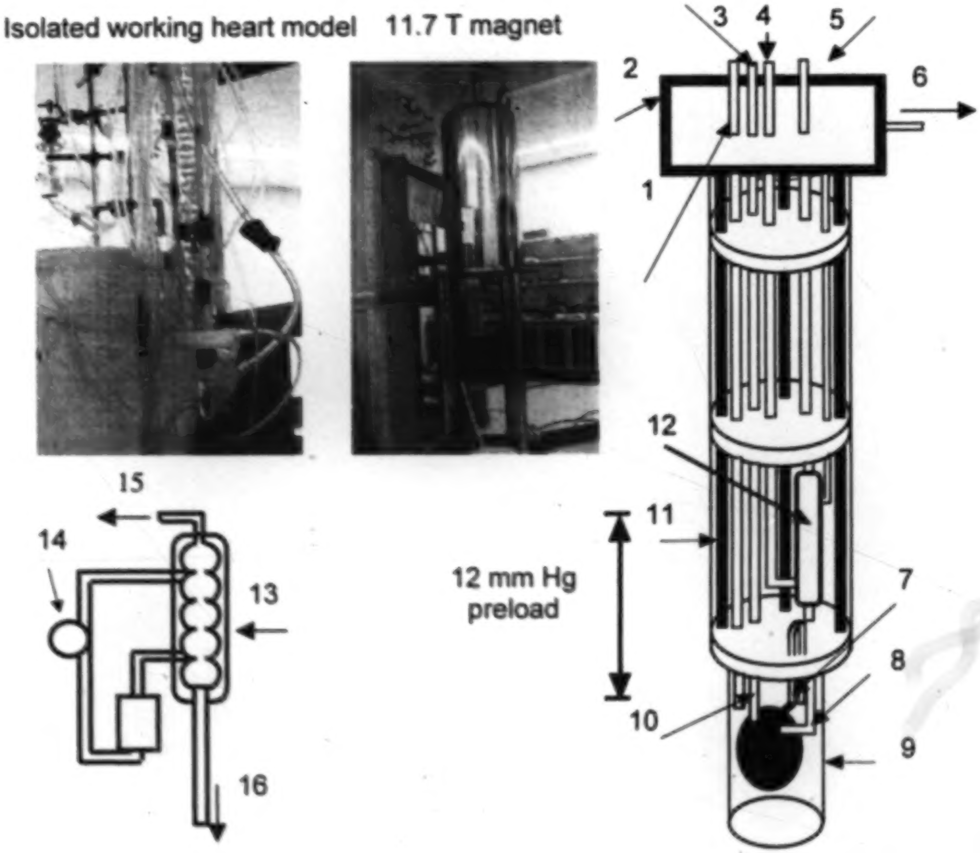
Isolated working heart model
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Figure 2-2. Original ^{31}P-NMR spectra under control conditions (A), after 20 min of ischemia (B) and after 25 min of reperfusion (C) in hearts perfused with glucose, palmitate and cariporide. pH$_i$ was determined from the shift difference between Pi and PCr. PCr resonance was set to 0 ppm.

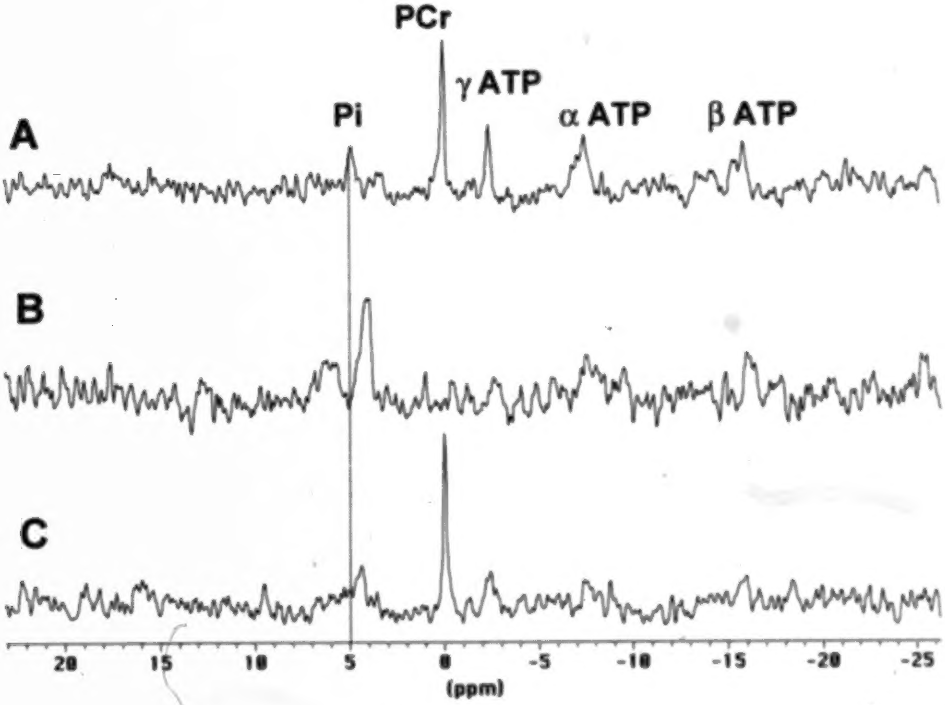

Table 2-1. Comparison of cardiac function between normal working rat heart model and working rat heart model for ^{31}P-NMR.

Parameter Measured	Normal working hearts (n=4)	Working hearts for ^{31}P-NMR (n=4)
Heart rate, beats • min^{-1}	246±8	233±8
Peak systolic pressure, mmHg	125±3	130±13
Developed pressure, mmHg	81±8	70±13
Cardiac output, ml •min^{-1}	49±3	50±4
Coronary flow, ml • min^{-1}	27±2	21±2

Values are means ± SEM of the number of hearts indicated. Hearts were perfused with glucose (5.5 mM) and palmitate (1.2 mM). Values were taken after 30 min of aerobic perfusion.

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Chapter 3

Acute effects of triiodothyronine on glucose and fatty acid metabolism during reperfusion of ischemic rat hearts

A version of this Chapter has been published

(Liu Q, et al. *Am J Physiol* 1998;275(38): E392-E399)

1. Introduction

It is well known that thyroid hormone regulates metabolic and physiological functions in cardiac tissue (see 1 for review). The direct effects of L-triiodothyronine (T_3), the biologically active form of the hormone, on cardiac cells can be nuclear or extranuclear in nature (see 1 for review). Nuclear effects are delayed in onset and are brought about by binding of thyroid hormone to nuclear thyroid hormone receptors. *In vivo*, hyperthyroidism can cause an increase in heart rate, contractility and cardiac output, thus raising the inotropic state of the heart which is accompanied by high level of energy substrate metabolism, especially glucose utilization[2,3]. Although these effects of thyroid hormone are thought to be the result of changes in myocardial gene expression, attention has recently focused on acute, non-nuclear mediated actions of T_3 (see 4 for review).

Extranuclear effects of thyroid hormone are rapid in onset, are not altered by inhibition of protein synthesis, and are mediated by thyroid hormone binding to plasma membrane receptors (see 4, 5 for review). Various lines of evidence have documented that T_3 can act as a vasodilator and positive inotrope *in vitro*[4,5]. The recognition of these effects has resulted in treatment strategies using T_3 that target specific clinical conditions associated with impaired cardiovascular performance and low serum T_3 concentration, including heart failure, cardiac surgery and acute myocardial infarction[1,4,6,7]. In clinical, as well as experimental trials, improved myocardial recovery in response to acute T_3 supplementation has been demonstrated after myocardial ischemia and cardiopulmonary bypass[7-9]. The mechanisms responsible for the cardioprotective effects of T_3 have yet to be defined.

In most clinical situations of reperfusion after ischemia, heart muscle is exposed to high levels of fatty acids (see 10 for review). When hearts are aerobically reperfused after ischemia, glucose oxidation is suppressed, due to high rates of fatty acid ß-oxidation. This results in a marked imbalance between rates of glycolysis and glucose oxidation[11,12]. In severely ischemic myocardium, production of protons (H^+) from the hydrolysis of glycolytically derived ATP is a major contributor to acidosis[3,13]. Clearance of H^+ via the NHE1 in aerobically perfused hearts subjected to an intracellular acid load leads to a significant decrease in cardiac efficiency[14,15]. This is due to the exchange of H^+ with extracelluar Na^+ via the NHE1. The intracellular Na^+ can then rapidly exchange with Ca^{2+} via the Na^+/Ca^{2+} exchanger during reperfusion, resulting in Ca^{2+} overload and cell death. Previous studies have shown that reducing the source of H^+ by stimulation of glucose oxidation or inhibition of excessive rates of glycolysis improves cardiac efficiency[15-18]. As a result, modifying glucose metabolism is one potential mechanism by which T_3 could potentially exert its cardioprotective effects. Previous studies have suggested that T_3 can modify both glycolysis and glucose oxidation in the heart[19,20]. Whether the cardioprotective effects of T_3 are attributable to changes in glucose metabolism or a switch in energy substrate preference has not been determined.

In this study we determined whether acute T_3 treatment could improve mechanical function and cardiac efficiency during reperfusion of ischemic hearts by modulation of glucose metabolism. Isolated working rat hearts perfused with a high level of fatty acid were subjected to a 30 min period of global no-flow ischemia, followed by 40 min of aerobic reperfusion. The effects of T_3 on the recovery of cardiac work, O_2 consumption (MVO_2), glycolysis, and oxidative metabolism of glucose and fatty acid were measured. Our results demonstrate that by reducing the production of H^+ from glucose metabolism, T_3 significantly improves the recovery of mechanical function and cardiac efficiency in the post-ischemic heart.

2. Methods (for general experimental procedure, please see Chapter 2 for details)

Glycolysis and glucose oxidation rates were measured simultaneously by perfusing hearts with [5-^{3}H/U-^{14}C]. Fatty acid oxidation rates were measured with perfusate containing [1-^{14}C]palmitate. TCA cycle activity was determined by calculating the rate of acetyl-CoA entering the TCA cycle from both glucose oxidation and fatty acid oxidation. Overall rates of H^+ production derived from glucose metabolism were determined by subtracting the rate of glucose oxidation from the rate of glycolysis and multiplying by two. PDH analysis was performed using a radioisotopic-coupled enzyme assay.

3. Experimental Protocol

Isolated working rat hearts were initially perfused for a 30 min period under aerobic conditions. Global no-flow ischemia was then introduced by clamping both the left atrial inflow and aortic outflow lines. After 30 min of no-flow ischemia, the left atrial and aortic flows were restored and the hearts were reperfused for a further 40-min period under aerobic conditions. T_3 was added at the onset of the 30 min aerobic working heart perfusion, at a final concentration of 10 nM (n=22). T_3 was diluted in 1 N NaOH (1 µl) immediately before use and the same amount of NaOH was added to the control group (n=17). Hearts were perfused with krebs-Henseleit solution containing 5.5 mM glucose, 1.2 mM palmitate and 100 µU • ml^{-1} insulin.

4. Results

4.1 Effects of T_3 on cardiac mechanical function of isolated working hearts subjected to 30 min of global no-flow ischemia

Fig. 3-1A shows the effects of treatment with 10 nM T_3 on the recovery of cardiac work in hearts subjected to 30 min of global ischemia. After severe ischemia, the recovery of cardiac work was depressed in control hearts, returning to only 11±3% of preischemic values after 40 min of reperfusion. During reperfusion, heart rate, peak systolic pressure, developed pressure, cardiac output, coronary flow were all significantly depressed compared to preischemic values. MVO_2 in control hearts recovered to a greater extent during reperfusion than did cardiac work (Table 3-1), resulting in a significant decrease in cardiac efficiency throughout the entire 40 min reperfusion period (Fig. 3-1B).

When 10 nM T_3 was added at the onset of the aerobic perfusion, there was no significant effect on the mechanical function of perfused hearts (Table 3-1, Fig. 3-1). However, at the end of 40 min of reperfusion, cardiac work recovered to 35±6% of preischemic values in T_3-treated hearts, compared with only 11±3% in control hearts ($p < 0.05$). Systolic pressure, developed pressure and cardiac output also recovered to significantly greater values than in control hearts (Table 3-1). T_3 had no effect on MVO_2 (Table 3-1), but due to the enhanced recovery of function, cardiac efficiency was increased during reperfusion compared with control (Fig. 3-1B).

Since T_3 has vasodilatory effects *in vitro* it is possible that the beneficial effects of T_3 could partly be explained by alterations in coronary flow. However, as shown in Table 3-1, T_3 did not significantly increase coronary flow during either the pre- or postischemia period. Furthermore, the increase in MVO_2 was less than the increase in contractile function postischemia, suggesting that the beneficial effect of T_3 seen in these hearts is not due to its vasorelaxant properties.

4.2 Effects of T_3 on glycolysis, glucose oxidation, and palmitate oxidation during reperfusion of hearts after ischemia

Fig. 3-2 and 3-3 show the time-dependent metabolism of glucose by glycolysis (panel A) and glucose oxidation (panel B) and palmitate oxidation. T_3 did not have any significant effects on glycolysis (Fig. 3-2A) and palmitate oxidation (Fig. 3-3), but did result in a significant increase in glucose oxidation during the reperfusion period (Fig. 3-2B). PDH activity (the rate limiting enzyme for glucose oxidation) was also measured in hearts frozen at the end of the reperfusion period. T_3-treatment significantly stimulated PDHa activity without affecting PDHt in post-ischemic hearts (Table 3-2). Although T_3 treatment stimulated glucose oxidation in the reperfusion period, no effect of T_3 on glucose oxidation was observed during the initial aerobic perfusion.

The effects of T_3 on steady state rates of glycolysis, glucose oxidation and palmitate oxidation are shown in Table 3-3. Steady state rates were calculated for values between 10 and 30 min of the aerobic period and between 10 and 40 min of the reperfusion period (Fig. 3-2). In control hearts, glycolysis recovered to pre-ischemic rates. T_3 had no significant effects on the rates of glycolysis during either the aerobic or reperfusion period. The steady state rate of glucose oxidation in control hearts was substantially lower than the rate of glycolysis (Table 3-3). This parallels previous observations in isolated working rat hearts perfused with high levels of fatty acid[8,12,15,21]. During reperfusion of control hearts, glucose oxidation did not recover to preischemic rates ($p < 0.05$). Treatment with T_3 resulted in a marked increase in the rate of glucose oxidation during reperfusion compared with control hearts ($p < 0.05$).

The steady state rate of palmitate oxidation during reperfusion of control hearts did not return to preischemic levels ($p < 0.05$). T_3 had no significant effect on palmitate oxidation rate during reperfusion compared with control hearts.

4.3 Effects of T_3 on H^+ production rate from glucose metabolism

Steady state H^+ production in aerobic and reperfused ischemic hearts is shown in Table 3-3. A significant decrease in H^+ production was seen in T_3-treated hearts, compared with control hearts. By selectively increasing the rate of glucose oxidation, T_3 improved the coupling between glycolysis and glucose oxidation, resulting in a significant decrease in H^+ production during reperfusion.

4.4 Effects of T_3 on rates of TCA cycle activity

To investigate TCA cycle activity during reperfusion, the rate of acetyl-CoA production from glucose oxidation and palmitate oxidation was calculated. As shown in Table 3-4, the total rate of TCA acetyl CoA production in control hearts was significantly decreased during reperfusion compared with preischemic values. This was consistent with the poor recovery of cardiac work. Treatment with T_3 did not alter overall acetyl-CoA production from glucose and palmitate oxidation. However, during reperfusion T_3 increased acetyl-CoA production from glucose.

5. Discussion

Acute treatment with physiologic or supraphysiologic concentrations of T_3 has been shown to have cardioprotective actions in experimental models of ischemia and reperfusion, as well as in the rescue of myocardial function after human cardiopulmonary bypass surgery[8-9,22]. Our data also show that acute T_3 can significantly improve the recovery of contractile function of isolated rat hearts subjected to a severe episode of no-flow ischemia. These effects of T_3 were associated with an improvement in the coupling of glycolysis to glucose oxidation, thereby decreasing H^+ production and increasing cardiac efficiency during reperfusion of the postischemic heart.

T_3 effects on cardiac energy metabolism

Few previous studies have determined directly the effects of acute T_3 treatment on energy metabolism in the heart. A study by Segal[20] showed that physiological concentrations of T_3 (1 pM to 10 nM) significantly stimulates 2-

deoxyglucose uptake in rat heart slices after as little as 10 min following treatment. However, in both aerobic and postischemic hearts, we observed that T_3-treatment was not associated with any significant effects on glycolysis. Therefore, we suggest that the cardioprotective effects of T_3 are not associated with an increase in glucose uptake and metabolism by glycolysis. Possible reasons for the differences between our study and that performed in rat heart slices are: 1) relevant level of fatty acid was present in our perfusate and not in the rat heart slice studies, and 2) hearts in our study were subjected to a physiological workload. Since circulating fatty acid levels are elevated both during and following clinically relevant conditions of ischemia, and fatty acids have dramatic effects on glucose metabolism, we felt it necessary to perform our studies in the presence of a relevant level of fatty acid. In addition, as rates of glucose metabolism are related to workload, all experiments were performed in hearts perfused in the working mode.

Our data show that the primary effect of T_3 on myocardial glucose metabolism is a stimulation of glucose oxidation during reperfusion. A previous study using rat cardiac myocytes also suggested that acute treatment of T_3 directly stimulates glucose oxidation[19]. The effects of T_3 on glucose oxidation are unlikely to be due to a generalized increase in oxidative metabolism, since the increase in glucose oxidation seen in T_3-treated hearts was not accompanied by an increase in fatty acid oxidation. Of interest is that the effects of T_3 on glucose oxidation were only observed during reperfusion of ischemic hearts, and not under aerobic preischemic conditions.

T_3 treatment significantly increased non-phosphorylated PDHa activity in postischemic hearts. Since PDHa plays an important role in regulation of glucose oxidation[23], our data strongly suggest that T_3 stimulates glucose oxidation and improves coupling of glycolysis to glucose oxidation secondary to a stimulation of PDHa activity. In rat hearts, ischemia or reperfusion have previously been shown to lead to an inactivation of PDHa under conditions similar to those used in the present study[24,25]. Ischemia is likely to increase intramitochondrial NADH/NAD$^+$ and acetyl-CoA/CoA ratios, which would lead to inactivation of PDH; however, the reduced ATP/ADP would balance this to some extent by favoring activation[24]. It has been reported that acute T_3 treatment can reduce intramitochondrial ATP/ADP[26,27], which may contribute to the observed activation of PDHa. However, the detailed mechanism whereby T_3 regulates PDH activity is still unclear. Future studies need to clarify whether acute T_3 treatment has any effect on PDH kinase or phosphatase, both of which also play an important role to regulate PDH activity[28,29]. PDH activity has also been shown to be stimulated by hyperthyroidism[30], although this is probably due to transcriptional regulation. Since acute treatment with T_3 is unlikely to up-regulate protein synthesis and PDHt was not altered, the observed effects of T_3 on PDHa in our study were likely the result of changes in the phosphorylated state of PDH.

Recovery of contractile function, energy metabolism and cardiac efficiency in the postischemic heart

During reperfusion of the severely ischemic control hearts, a significant decrease in the recovery of cardiac function occurred which was associated with a decrease in cardiac efficiency (Fig. 3-1). This decrease in cardiac efficiency has also been observed in previous studies[11,15]. However, unlike these previous studies, we did not observe a complete recovery of fatty acid oxidation in control hearts during reperfusion. This difference in the recovery of fatty acid oxidation may be related to the severity of ischemic injury observed in the present study. As shown in Fig. 3-1, cardiac work recovered to 11±3% of the preischemic value in control hearts, compared to 30-40% in our previous studies[11,15]. However, it should be recognized that despite this poor recovery of cardiac work, fatty acid oxidation in control hearts recovered to over 50% of preischemic levels, resulting in a marked increase in fatty acid oxidation per unit work, a finding consistent with our previous studies. Regardless of the degree of recovery of fatty acid oxidation in this study, our data suggest that the beneficial effects of T_3 are unlikely to be due to any direct effects on fatty acid oxidation.

It is well known that long-term hyperthyroidism is associated with high levels of MVO_2. However, with acute treatment of T_3, this is not the case. A recent study by Klemperer *et al*[22] showed that acute T_3 treatment improves left ventricular function following ischemia without oxygen-wasting effects in isolated rat hearts. In the present study, we also found that T_3 significantly improves the recovery of cardiac work without a concomitant increase in MVO_2. Therefore, an improved cardiac efficiency (ratio of cardiac work to MVO_2) was observed in the postischemic heart.

Previous studies[8] suggest that combination of global ischemia and depletion of T_3 results in reduced mitochondrial function, inhibition of TCA cycle activity and increased anaerobic metabolism. T_3 replacement therapy leads to improved mitochondrial function and increased anaerobic metabolism. In the present study, we observed that acetyl-CoA production from glucose and fatty acid oxidation was significantly inhibited after 30 min of severe ischemia, whereas glycolysis was unaffected. Treatment with T_3 dramatically increased acetyl-CoA production from glucose oxidation with minor effects on fatty acid oxidation (Table 3-4). This suggests that T_3 may directly affect mitochondrial function. Overall acetyl-CoA production was not significantly increased, which suggests that T_3 has no effect on the efficiency of energy production. Rather, T_3 improves the efficiency of energy utilization. Thyroid hormone exerts two types of effects on mitochondria (see 31 for review). Within a few minutes of administration, T_3 causes a rapid activation of respiration, an effect that is preserved in isolated mitochondria. A direct control of oxidative phosphorylation through binding of T_3 to mitochondria is thought to occur[31-33]. This control of oxidative phosphorylation is observed after administration of physiologic amounts of T_3 and is not altered by inhibition of protein synthesis[26,31]. The mitochondrial binding site has been proposed to be the adenine nucleotide translocase[32,33], an inner mitochondrial membrane carrier that catalyzes exchange between the extra- and intramitochondrial ADP and ATP[26,34,35]. Of interest is the shift of the mitochondria ATP/ADP ratio also involved in the regulation of PDH activity, thereby controlling pyruvate oxidation and consequently glucose oxidation[26,27]. However, studies identifying the adenine nucleotide translocase as a T_3 receptor (as well as the role of adenine nucleotide translocase in short-term activation of respiration) have been questioned (see 31 for review), and further studies are necessary to clarify this possibility.

In our study, the actual measured TCA cycle rates were lower than predicted TCA cycle rates calculated from measured oxygen consumption values shown in Fig. 3-1. This is likely the result of the significant proton leak that occurs in the mitochondrial membrane[36]. Depending on the extent of proton leak, oxygen consumption rates can substantially overestimate actual TCA cycle activity. This raises the possibility that T_3 may increase cardiac efficiency by decreasing the mitochondrial proton leak. However, in this study we measured both parameters, and found that T_3 effects on TCA cycle activity (Table 3-4) and oxygen consumption during reperfusion of ischemic hearts (Fig. 3-1) were comparable. That is, T_3 increased TCA cycle during reperfusion by 27% (from 5.6 ± 0.5 to 7.1 ± 0.8 μmol • g dry wt^{-1} • min^{-1}) and oxygen consumption by 30% (from 22.6 ± 6.7 to 29.4 ± 5.5 μmol • g dry wt^{-1} • min^{-1}). This suggests that T_3 does not act by altering the mitochondrial H^+ leak, and supports our hypothesis of an increased cardiac efficiency secondary to decreasing proton production from glycolysis uncoupled from glucose oxidation.

Coupling of glycolysis to glucose oxidation in the postischemic heart

The production of H^+ from glucose metabolism is an important contributor to the impaired recovery of mechanism function and to the decrease in cardiac efficiency seen following a severe ischemic episode[11,12,15]. During reperfusion, treatment with T_3 dramatically stimulated glucose oxidation, with no effect on glycolysis. Each molecule of glucose that passes through glycolysis that is not subsequently oxidized results in the production of 2 H^+ from the hydrolysis of glycolytically derived ATP[11,15]. In the presence of high levels of fatty acids, glucose oxidation rates are 5-fold to 10-fold lower than glycolytic rates[11,12,37]. Selective stimulation of glucose oxidation improves the coupling of glycolysis to glucose oxidation, leading to a reduction in H^+ production. We have suggested that an increase in H^+ accumulation during the critical period of reperfusion may contribute to cardiac inefficiency[10] and the well-documented Ca^{2+} overload in the postischemic heart that occurs due to an increase in NHE1 activity coupled with Na^+/Ca^{2+} exchange[14]. Because T_3 reduced the H^+ production from glucose utilization during reperfusion the driving force for the NHE1 is decreased, and Na^+-Ca^{2+} exchange activity would thus be expected to be reduced during reperfusion. Decreased activity of this exchanger may be responsible for the significant improvement in cardiac efficiency observed during reperfusion.

A number of other pharmacological agents also stimulates glucose oxidation and has a beneficial effect on the recovery of mechanical function during reperfusion of the postischemic heart [16,37]. One of these agents is DCA, a potent PDH activator, which also improves the coupling between glycolysis and glucose oxidation. This also results in a significant decrease in H^+ production from glucose metabolism during reperfusion, resulting in a significant increase in cardiac efficiency.

Summary

We demonstrate a significant improvement of recovery in postischemic cardiac function and efficiency in isolated rat hearts using moderately supraphysiological amounts of T_3. The cardioprotective effect of T_3 may be due to its stimulation of glucose oxidation, secondary to an increase in PDHa activity, and therefore a reduction in the production of H^+ by coupling glycolysis with glucose oxidation.

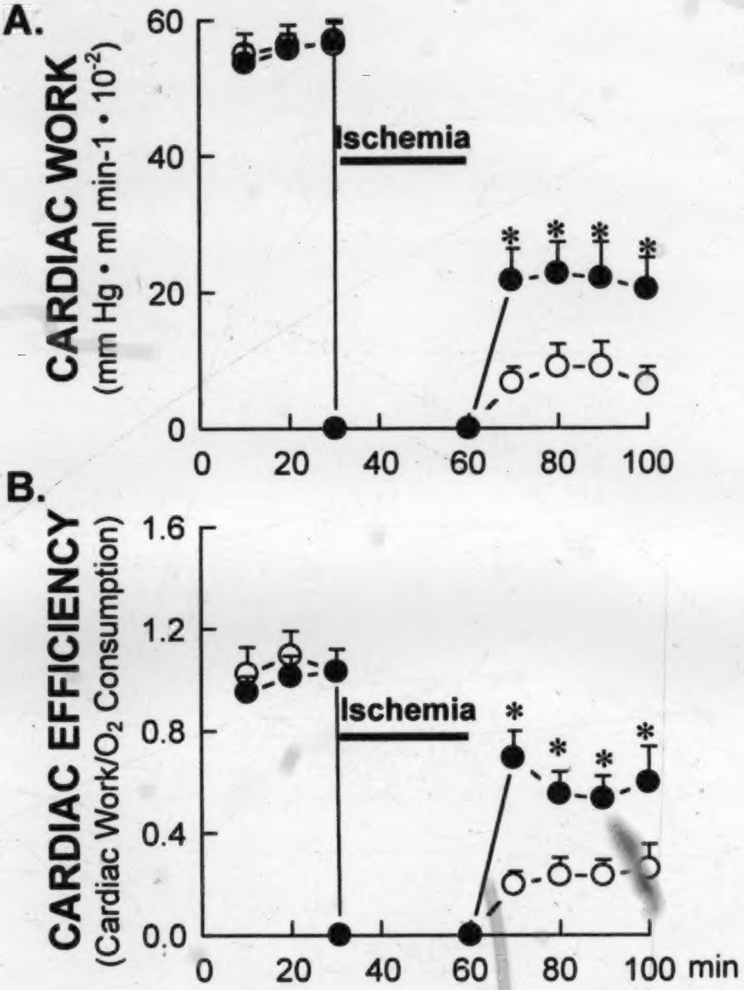

Figure 3-1. Effects of T_3 on the recovery of cardiac work (A), and cardiac efficiency (B) of isolated working hearts subjected to 30 min of global no-flow ischemia. Values are mean ± SEM of 17 control hearts (O) and 22 hearts T_3 (10 nM)-treated hearts (●).

*, Significantly different from control hearts.

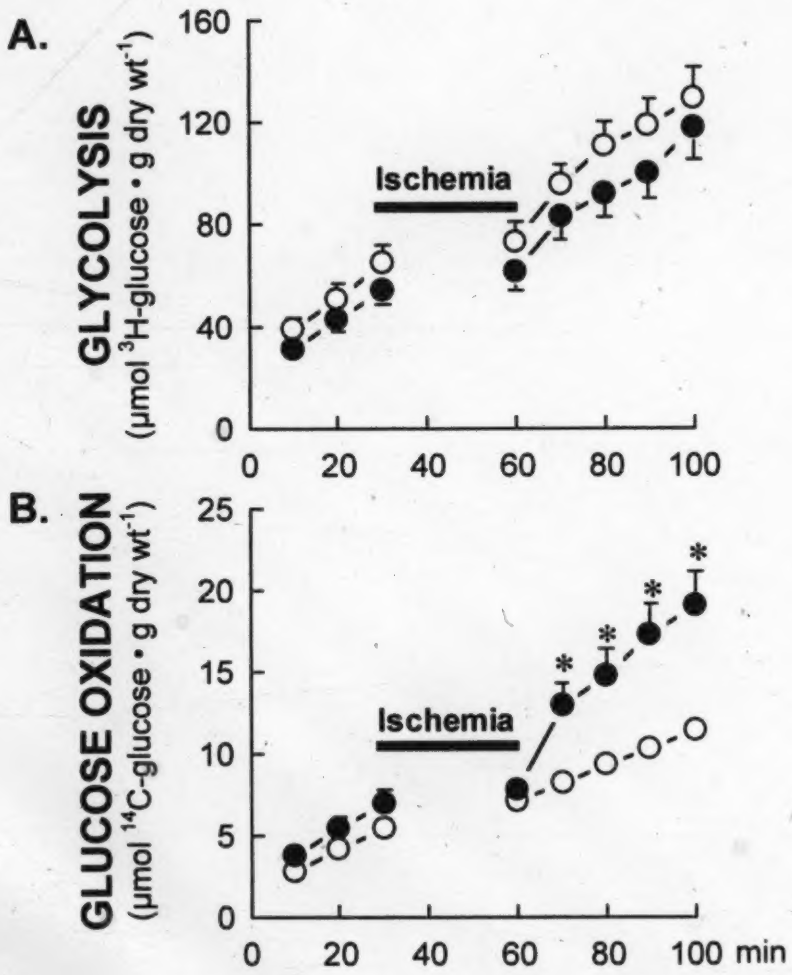

Figure 3-2. The effects of T_3 on the time course of glycolysis (A), glucose oxidation (B), in hearts reperfused after 30 min of global no-flow ischemia. Values are mean ± SEM. In panel A, control (O) n=12, T_3 (●) n=16; In panel B, control (O) n=12, T_3 (●) n=16. *, Significantly different from control hearts.

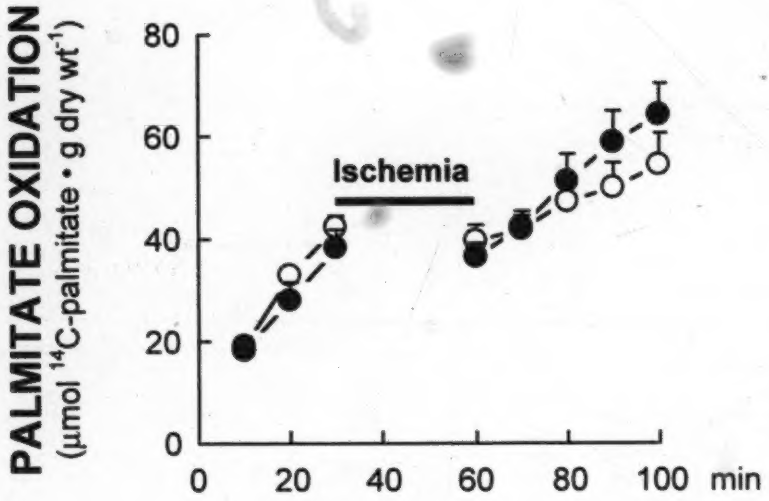

Figure 3-3. The effects of T_3 on the time course of palmitate in hearts reperfused after 30 min of global no-flow ischemia. Values are mean ± SEM. control (O) n=5, T_3 (●) n=7.

Table 3-1. Effects of T_3 on the recovery of mechanical function of postischemic working rat hearts.

Parameter measured	Control (n=17)	T_3 treated (n=22)
Aerobic		
Heart rate (beats • min^{-1})	246 ± 8	226 ± 5
Peak systolic pressure (mm Hg)	117 ± 3	110 ± 2
Developed pressure (mm Hg)	81 ± 8	79 ± 8
MVO_2 (µmol glucose • dry wt^{-1})	58 ± 4	59 ± 3
Cardiac Output (ml • min^{-1})	49 ± 3	51 ± 2
Coronary flow (ml • min^{-1})	27 ± 2	26 ± 2
Reperfused after Ischemia		
Heart rate (beats • min^{-1})	96 ± 17^{+}	126 ± 15^{+}
Peak systolic pressure (mm Hg)	30 ± 7^{+}	59 ± 9^{+*}
Developed pressure (mm Hg)	4 ± 7^{+}	17 ± 15^{+*}
MVO_2 (µmol glucose • dry wt^{-1})	23 ± 7^{+}	29 ± 5^{+}
Cardiac output (ml • min^{-1})	10 ± 3^{+}	20 ± 4^{+*}
Coronary flow (ml • min^{-1})	8 ± 3^{+}	11 ± 2^{+}

$^{+}$ Significantly different from preischemic values.

* Significantly different from postischemic values in control hearts.

Table 3-2. Effect of T_3 on active and total pyruvate dehydrogenase activity in hearts reperfused following ischemia.

	PDHa (nmol g dry wt^{-1} min^{-1})	PDHt (nmol g dry wt^{-1} min^{-1})	PDHa/PDHt (%)
Control	2300 ± 508	9578 ± 664	23 ± 4
T_3	3245 ± 439*	9585 ± 507	33 ± 4*

Hearts were subjected to 30 min of aerobic perfusion, 30 min of global no-flow ischemia and 40 min of aerobic reperfusion. Values are mean ± SEM of 11 hearts in each group. T_3 (10 nM) was added at the onset of aerobic perfusion.

* Significantly different from control hearts.

Table 3-3. Effects of T_3 on steady state rates of glycolysis, glucose oxidation, and H^+ production from glucose utilization before and after ischemia.

Parameter measured (nmol • g dry wt^{-1} • min^{-1})	Control	T_3
Aerobic		
Glycolysis	1270±190 (9)	1185±183 (13)
Glucose oxidation	160±18 (9)	142±17 (13)
H^+ production	2220±100 (9)	2086±365 (13)
Palmitate oxidation	1350±220 (5)	1140±106 (7)
Reperfused after Ischemia		
Glycolysis	1465±153 (9)	1163±158 (13)
Glucose oxidation	95±8[✦] (9)	230±29[•] (13)
H^+ production	2740±310 (9)	1867±311[•] (13)
Palmitate oxidation	680±70[✦] (5)	823±100 (7)

Values are means ± SEM of the numbers shown in the brackets.

[✦] Significantly different from preischemic values.

[•] Significantly different from postischemic values in control hearts.

Table 3-4. Effects of T_3 on the source of tricarboxylic acid cycle acetyl-CoA production from glucose and fatty oxidation in aerobic and postischemic hearts.

Source of Acetyl-CoA (μmol • g dry wt^{-1} • min^{-1})	Control	T_3 treated
Aerobic		
From Glucose Oxidation	0.32±0.04 (9)	0.28±0.03 (13)
From Palmitate Oxidation	10.8±1.7 (5)	9.1±0.8 (7)
Total TCA Cycle Activity	11.1±1.7	9.4±0.8
Reperfused after Ischemia		
From Glucose Oxidation	0.19±0.02^{+} (9)	0.46±0.06^{+*} (13)
From Palmitate Oxidation	5.4±0.5^{+} (5)	6.6±0.8 (7)
Total TCA cycle activity	5.6±0.5	7.1±0.8

* Significantly different from postischemic values in control group.

$^{+}$ Significantly different from preischemic values.

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Chapter 4

High levels of fatty acids delay the recovery of pH_i and cardiac efficiency in postischemic hearts by inhibiting glucose oxidation

(A version of this Chapter has been submitted for publication in *Circulation Research* 2000)

1. Introduction

During ischemia, anaerobic glycolysis is an important source of ATP production[1]. However, H^+ production from the hydrolysis of glycolytically produced ATP can also become a major contributor to the acidosis that occurs in severely ischemic myocardium[2]. Development of severe cellular acidosis decreases cardiac pressure development[3] and provokes cardiac arrhythmias[4]. How either of these processes is mediated has not been fully elucidated. Acidosis decreases the response of contractile proteins to Ca^{2+}, predominantly by a decrease in Ca^{2+} binding to troponin C as H^+ compete with Ca^{2+} at Ca^{2+}-binding sites[4]. Intracellular acidosis during severe ischemia also increases sarcolemmal Na^+/H^+ exchange (NHE1)[5,6]. If the myocardium is reperfused, extracellular pH quickly normalizes, creating a large pH gradient across the membrane, and the resultant activation of NHE1 increases intracellular Na^+. Accumulation of intracellular Na^+ may activate Na^+/Ca^{2+} exchanger and lead to intracellular Ca^{2+} overload and cell death[5,6]. Inhibition of NHE1 can dramatically improves the recovery of cardiac function and efficiency during reperfusion[7-9], emphasizing that the accumulation of intracellular H^+ during ischemia is an important contributing factor to reperfusion injury. Whether continued production of H^+ during the critical early period of reperfusion also has the potential to exacerbate ischemia-reperfusion injury has not been established.

In most clinical situations of reperfusion after ischemia, heart muscle is exposed to high levels of fatty acids[10] and reperfusion of reversibly injured ischemic muscle under this condition results in a rapid recovery of fatty acid oxidation, with rates often exceeding preischemic levels[11,12]. This high rate of fatty acid oxidation inhibits the rate of glucose oxidation to a much greater extent than the rate of glycolysis which results in a marked uncoupling between rates of glycolysis and glucose oxidation[13-16]. This uncoupling of glucose metabolism is a potentially important source of H^+ production during reperfusion[13,15,16]. If glycolysis is coupled to glucose oxidation, H^+ production from hydrolysis of ATP derived from glucose metabolism is zero[13,17]. However, if glycolysis is uncoupled from glucose metabolism (and pyruvate derived from glycolysis is not oxidized), there is a net production of $2H^+$ from each glucose molecule metabolized. As a result, high rates of fatty acid oxidation during the actual reperfusion period have the potential to increase H^+ production from uncoupled glucose metabolism[13-16]. This increase in H^+ production during reperfusion is associated with a poor recovery of cardiac function and efficiency. Furthermore, reduction of H^+ production by either inhibiting glycolysis or stimulating glucose oxidation can improve the recovery of cardiac function and efficiency during reperfusion[15,16]. On the basis of these findings we speculate that high levels of fatty acids decrease cardiac function and efficiency during reperfusion by increasing the production of H^+ during the critical early period of reperfusion. Although there is evidence to indicate that fatty acid increases the calculated rate of H^+ production during reperfusion[13-16], direct evidence demonstrating that a high level of fatty acid alters the rate of recovery of pH_i following ischemia is lacking.

Measurement of pH_i can be achieved with ^{31}P-nuclear magnetic resonance (^{31}P-NMR), which is considered to be the gold standard methodology for many years. However, to our knowledge no previous studies have used this technique to assess directly the effects of fatty acids on rates of pH_i recovery in working hearts following ischemia. In this study, we used ^{31}P-NMR to measure directly pH_i in isolated working rat hearts in the presence and absence of a high level of fatty acid. Using this approach we were able to compare rates of recovery of pH_i following an episode of severe ischemia with rates of H^+ production calculated from measurements of glycolysis and glucose oxidation. We also determined whether stimulation of glucose oxidation with dichloroacetate (DCA), a pyruvate dehydrogenase activator[14,18], could improve the recovery of cardiac function and efficiency by accelerating the recovery of pH_i during reperfusion.

2. Methods (for general experimental method, please see Chapter 2 for details)

Glycolysis and glucose oxidation rates were measured simultaneously by perfusing hearts with [5-^{3}H/U-^{14}C]. Overall rates of H^+ production derived from glucose metabolism were determined by subtracting the rate of glucose oxidation from the rate of glycolysis and multiplying by two. pH_i and high energy phosphate were measured by ^{31}P-NMR.

3. Experimental protocol

Isolated working rat hearts were subjected to 30 min aerobic perfusion (37 °C), 20 min of global no-flow ischemia (33 °C) and 40 min of reperfusion (37 °C). Experimental groups were: 1) 5.5 mM glucose throughout (Glucose), n=8, 2) 5.5 mM glucose and 1.2 mM palmitate throughout (Glucose+Palmitate), n=8 or 3) 5.5 mM glucose and 1.2 mM palmitate throughout with 3 mM DCA added immediately before postischemic reperfusion (Glucose+Palmitate+DCA), n=8. Hearts were perfused with Krebs-Henseleit solution containing 5.5 mM glucose, 1.2 mM palmitate.

4. Results

4.1 Effects of palmitate on baseline cardiac function

The effects of a high level of fatty acid (1.2 mM palmitate) on various measures of cardiac function in aerobic and reperfused ischemic hearts are shown in Table 4-1 and Fig. 4-1. Baseline preischemic values for heart rate, peak systolic pressure, developed pressure, cardiac output, cardiac work, MVO_2, coronary flow and coronary vascular resistance (CVR) did not differ between Glucose+Palmitate and Glucose hearts. Baseline pH_i values in Glucose+Palmitate and Glucose hearts were also not different after 30 min aerobic perfusion (7.16±0.02 and 7.14±0.03, respectively, $p>0.05$).

4.2 Effects of palmitate on the recovery of cardiac function and efficiency

Fig 4-1A shows the effects of palmitate on the recovery of cardiac work in hearts subjected to 20 min of global ischemia. This period of ischemia was chosen since it resulted in a near complete recovery of cardiac work in the Glucose group (83±8% of preischemic values). Similarly, MVO_2 recovered to preischemic level (94±5%)However, in Glucose+Palmitate hearts the recovery of cardiac work was dramatically depressed, returning to only 30±8% of preischemic values after 40 min of reperfusion. Heart rate, peak systolic pressure, developed pressure, cardiac output, and coronary flow were all significantly depressed during reperfusion in Glucose+Palmitate hearts compared with Glucose hearts (Table 4-1). However, CVR was not different between these two groups. In contrast, in the Glucose+Palmitate hearts MVO_2 recovered to a greater extent during reperfusion (Table 4-1) than cardiac work (Figure 4-1A), resulting in a significant decrease in cardiac efficiency throughout the entire 40-min reperfusion period (Fig 4-1B).

4.3 Effects of palmitate on glucose metabolism and H^+ production

Fig.4-2 shows amount of substrate metabolized versus time by glycolysis (panel A) or glucose oxidation (pane B) throughout the entire perfusion period. Rates of both glycolysis and glucose oxidation were linear in both the Glucose+Palmitate and Glucose hearts during the preischemic and postischemic periods. There was no difference in glycolysis and glucose oxidation between Glucose+Palmitate and Glucose hearts during preischemic period, respectively. Steady-state rates, calculated during the postischemic period, are shown in Table 4-2. No significant difference in glycolysis rates were observed during reperfusion between the Glucose+Palmitate and Glucose hearts. However, glucose oxidation was significantly lower in Glucose+Palmitate hearts compared with Glucose hearts. This resulted in a substantial uncoupling of glycolysis from glucose oxidation, leading to a significantly higher calculated rate of H^+ production from glucose metabolism during reperfusion in the Glucose+Palmitate hearts.

4.4 Effects of palmitate on the recovery of pH_i

The effects of palmitate on pH_i and high energy phosphates during ischemia and reperfusion periods are shown in Fig.4-3. As expected, pH_i decreased during ischemia, but there was no difference in pH_i between the Glucose+Palmitate and the Glucose hearts.

At the onset of reperfusion, pH_i began to recover in both groups. In Glucose hearts, recovery of pH_i was rapid and after 5 min of reperfusion, pH_i had recovered to preischemic values. In contrast, pH_i recovered slowly in the Glucose+Palmitate hearts to preischemic values after 40 min of reperfusion. Three phases of recovery of pH_i were seen in Glucose+Palmitate hearts. Within the first 3 min of reperfusion, there was a rapid recovery of pH_i in both groups and there was no difference in the rate of recovery of pH_i between Glucose and Glucose+Palmitate groups (0.11 ± 0.02 vs 0.10 ± 0.03 pH units • min^{-1}, respectively, $p>0.05$). In the Glucose group pH_i quickly recovered to a preischemic level after a further 2 min of reperfusion, while pH_i in the Glucose+Palmitate group recovered slowly during the next 27 min of reperfusion. The rate of recovery of pH_i in the Glucose+Palmitate group was significantly slower (0.01 ± 0.005 vs 0.16 ± 0.04 pH units • min^{-1}, in Glucose+Palmitate hearts and Glucose hearts, respectively, $p<0.05$). Within the final 10 min of reperfusion (30 to 40 min), the pH_i in Glucose+Palmitate hearts quickly recovered to the preischemic level. The rate of pH_i recovery in the Glucose+Palmitate group for the last 10 min of reperfusion was significantly higher than that for the previous 27 min of reperfusion (0.02 ± 0.003 vs 0.01 ± 0.005 pH units • min^{-1}, $p<0.05$).

4.5 Effects of DCA on the recovery of cardiac function and efficiency

To determine if stimulating glucose oxidation could overcome the detrimental effects of palmitate on cardiac function and pH_i recovery, DCA was added to the perfusate at the onset of reperfusion in Glucose+Palmitate hearts[14,18]. Fig 4-4 shows the effects of DCA on the recovery of cardiac work (A) and cardiac efficiency (B) during reperfusion following ischemia. Similar to the results from Fig.4-1, the recovery of cardiac work was dramatically depressed in the Glucose+Palmitate group and returned to only 38±3% of preischemic values after 40 min of reperfusion. Heart rate, peak systolic pressure, developed pressure, cardiac output, and coronary flow were also significantly depressed in these hearts during the postischemic period (Table 4-3), as was cardiac efficiency (Fig. 4-4B). When DCA was present during reperfusion, cardiac work recovered to 74±6% of preischemic values by the end of reperfusion, which was significantly greater compared with 38±3% in untreated hearts ($p<0.05$). Heart rate, peak systolic pressure, developed pressure, cardiac output, coronary flow also recovered to a greater extent during reperfusion in DCA (Table 4-3). However, there was no difference in the recovery of CVR between Glucose+Palmitate and Glucose+Palmitate+DCA hearts. Although the recovery of MVO_2 was also significantly improved in DCA-treated hearts, the improvement in cardiac efficiency observed in DCA-treated hearts was significantly greater compared with untreated hearts, due to the greater extent of recovery of cardiac work.

4.6 Effects of DCA on glucose metabolism, H^+ production from glucose metabolism and pH_i recovery following ischemia

Fig.4-5 shows amount of substrate metabolized versus time by glycolysis (panel A) or glucose oxidation (panel B) throughout the entire perfusion period. Steady-state rates of glycolysis, glucose oxidation and H^+ production during reperfusion are shown in Table 4-4. During reperfusion, DCA increased the rate of glucose oxidation by 316%, with no significant effect on the glycolytic rate, resulting in a 35% decrease in H^+ production ($p<0.05$).

The effect of DCA on pH_i during the reperfusion period is shown in Fig. 4-6. When DCA was present during reperfusion, a significant increase in the rate of recovery of pH_i following ischemia was observed, and complete recovery of pH_i was seen after 10 min of reperfusion. In contrast, pH_i recovered slowly in the Glucose+Palmitate hearts and reached preischemic values only after 40 min of reperfusion. Within the first 3 min of reperfusion, there was a rapid recovery of pH_i in both groups and there was no difference in the rate recovery of pH_i between Glucose+Palmitate+DCA and Glucose+Palmitate groups (0.10 ± 0.03 vs 0.10 ± 0.04 pH units • min^{-1}, respectively, $p>0.05$). After a further 7 min of reperfusion pH_i in DCA group had recovered to the preischemic level, while pH_i in Glucose+Palmitate group recovered slowly after a further 27 min of reperfusion. The rate of pH_i recovery in the Glucose+Palmitate group was significantly slower than the DCA-treated hearts (0.01 ± 0.003 vs 0.07 ± 0.005 pH units • min^{-1}, in Glucose+Palmitate hearts and Glucose+Palmitate+DCA hearts, respectively, $p<0.05$). Within the final 10 min of reperfusion, the pH_i in Glucose+Palmitate hearts recovered to the preischemic level, the rate of recovery of pH_i being significantly higher than for previous 27 min of reperfusion (0.02 ±0.005 vs 0.01±0.003 pH units • min^{-1}, $p<0.05$).

4.7 Effects of palmitate and DCA on the recovery of energy phosphates

Phosphocreatine (PCr), ATP, and inorganic phosphate (Pi) contents during ischemia and reperfusion in the Glucose, Glucose+Palmitate, and Glucose+Palmitate+DCA hearts are shown in Fig.4-7. During ischemia, PCr and ATP contents decreased, while Pi content increased, and there were no difference in above energy phosphate contents among these three groups. Recovery of PCr content during reperfusion was greatest in Glucose+Palmitate+DCA hearts, while recovery of PCr content in Glucose+Palmitate hearts was significantly poorer compared with Glucose hearts, in which PCr content recovered to the preischemic level. There was no significant difference in ATP or Pi content among these three groups during reperfusion, although a tendency for a better recovery of ATP in Glucose hearts was observed.

5. Discussion

A growing body of literature supports the notion that myocardial energy substrate preference is an important determinant of the ability of cardiac muscle to recovery following an ischemic episode[15,16,19,21]. In this study we confirm previous studies showing that a high level of fatty acid (which is seen in most clinically relevant conditions of ischemia) markedly inhibits glucose oxidation during reperfusion of ischemic hearts[14-16]. An important novel finding is that the resulting calculated increase in H^+ production from glycolysis uncoupled from glucose oxidation does indeed delay the recovery of pH_i during reperfusion. This contributes to a fatty acid-induced decrease in recovery of both mechanical function and cardiac efficiency during reperfusion. The second important finding in this study is that directly stimulating glucose oxidation in fatty acid perfused hearts (with DCA), accelerates the rate of recovery of pH_i during reperfusion, secondary to a decrease in H^+ production from glucose metabolism. Confirming our previous studies, this stimulation of glucose oxidation is accompanied by an significant improvement in mechanical function and cardiac efficiency during reperfusion[14-16]. Of interest, is the demonstration that the beneficial effects of stimulating glucose oxidation occurred during the actual reperfusion period. It is well known that H^+ accumulation during ischemia is an important contributing factor to ischemic injury (see reference 2,21 for reviews). Our results demonstrate that the continued production of H^+ during the actual reperfusion period also contributes to cardiac injury.

Advantages of experimental approach

Despite widespread recognition that ^{31}P-NMR is an effective approach to measure pH_i in the heart, only a few studies have used this technique *in vitro* to measure pH_i during and following ischemia in hearts perfused without the high levels of fatty acid seen *in vivo* during and following ischemia. To our knowledge, no previous study has specifically investigated the effects of a high level of fatty acid on rates of recovery of pH_i, nor has pH_i recovery been directly compared with calculated rates of H^+ production from glycolysis and glucose oxidation. Since metabolic rates are highly dependent on the work performed by the heart, we developed techniques that allowed the simultaneous measurement of mechanical function and pH_i in isolated working rat hearts perfused in the presence of a high level of fatty acid (1.2 mM palmitate). Although intensive modifications of the perfusion system were required, cardiac mechanical function in this study was not different to those of our previous studies (see Chapter 2 for details). Moreover, during an episode of global no-flow ischemia, the decrease in pH_i in glucose-perfused hearts was similar to the decrease observed in numerous previous studies [8,22-24].

Effects of palmitate on H^+ production and recovery of pH_i, cardiac function and efficiency

Although a considerable research effort has concentrated on the fate of H^+ during reperfusion of ischemic hearts, little attention has focused on whether H^+ production during reperfusion contributes to ischemic injury. It is well recognized that hydrolysis of glycolytically produced ATP is a major source of acidosis in the severely ischemic heart[1,2]. During ischemia, oxidative metabolism is decreased and pyruvate produced by glycolysis is directed towards lactate production. This results in the production of two H^+ for every glucose molecule metabolized by glycolysis[2]. During reperfusion of ischemic hearts oxidative metabolism recovers, but high rates of fatty acid oxidation result in low rates of glucose oxidation[13-16]. As shown in Table 4-2, the marked inhibition of glucose oxidation in the presence of a high level of fatty acid is not accompanied by a similar decrease in glycolytic rates. As a result, glycolysis is further uncoupled from glucose oxidation and continues to be an important source of H^+ during the actual reperfusion period. Our data strongly suggest that this is responsible for the slower rate of recovery of pH_i in Glucose+Pamitate hearts during reperfusion.

Interestingly, during ischemia, the presence of high levels of fatty acids had no effect on the rate or extent of the decrease in pH_i. During early phase of reperfusion (0 to 3 min), there was a rapid recovery of pH_i in Glucose, Glucose+Palmitate and Glucose+Palmitate+DCA hearts. This may be because intracellular H^+ were quickly washed out during the restoration of flow. In the late period of reperfusion, the marked slower recovery of pH_i in Glucose+Palmitate group may be due to fatty acid-induced increase in H^+ production, as opposed to an alteration in the fate of the H^+ produced. Our data also suggest that this increased H^+ burden contributes to the decrease in cardiac work and cardiac efficiency during reperfusion, since preventing the fatty acid-induced increase in H^+ production (by stimulating glucose oxidation or by omitting fatty acids) improved the recovery of both cardiac work and cardiac efficiency. Furthermore, our data in Chapter 5 show that inhibition of NHE1 by cariporide leads to a complete recovery of postischemic cardiac mechanical function and efficiency in hearts perfused with both glucose and palmitate (see Chapter 5 for details), indicating that the increased H^+ production activates NHE1 and causes ischemia-reperfusion injury. Worthy of mention, during the final 10 min of reperfusion (30 to 40 min), pH_i in Glucose+Palmitate hearts recovered quickly to the preischemic level and the rate of recovery of pH_i was significantly higher than for the previous 27 min of reperfusion. This may be because there are at least four different transports that contribute to the recovery of pH_i in the heart: NHE1[7], lactate-H^+ co-transporter (MCT)[25], vacuolar–H^+ ATPase[26,29] and Na^+/HCO_3^{-1} co-transporter[27]. It is possible that at least one of these H^+ extrusion pathways is hyperactivated and accelerates the recovery of pH_i.

While the high level of fatty acid decreased the rate of pH_i recovery following ischemia in our study, this may not suggest it is the rate of pH_i recovery *per se* that is responsible for the detrimental effects of fatty acids. Rather, our evidence indicates that it is the H^+ accumulation in the heart as well as the fate of this increased H^+ load that contributes to injury. Previous studies have also shown that NHE1 inhibitors slow the rate of recovery of pH_i following ischemia [8,24,31,37], although recovery of cardiac function and cardiac efficiency following ischemia is improved[28,37]. As discussed above, inhibition of NHE1 prevents intracellular Na^+ accumulation, and although H^+ clearance rates are decreased, it is thought that, in the presence of NHE1 inhibitors, H^+ are directed towards clearance pathways that do not lead to Na^+ and Ca^{2+} overload[26]. If this is the case, then decreasing the actual production of H^+ should have similar beneficial effects as NHE1 inhibitors on cardiac function and efficiency. which is what we observed in this study. Furthermore, additional data (see Chapter 5 for details) also show that in the presence of specific NHE1 inhibitor, cariporide, treatment with DCA did not further improve the recovery of cardiac function and efficiency, although the rate recovery of pH_i was synergistically accelerated during reperfusion. However, it remains to be determined whether fatty acids affect intracellular Na^+ and Ca^{2+} concentration during reperfusion, or whether inhibiting fatty acid-induced H^+ production from glucose metabolism decreases Na^+ and Ca^{2+} overload following ischemia.

Relationship between accumulation of H^+ production and pH_i

pH_i is the consequence of accumulated H^+ load that overcomes the intracellular H^+ buffering capacity (see 30 for review). Therefore, the concentration of accumulated H^+ production calculated from glucose metabolism should be higher than pH_i. For example, in Glucose+Palmitate group, pH_i value at the first 10 min of reperfusion was 6.83±0.06 (0.15 μM). The H^+ production rate in Glucose+Palmitate group was 1136±85 $\mu mol \cdot g\ dry\ wt^{-1} \cdot min^{-1}$, which was equal to concentration of H^+-2.2 mM after 10 min reperfusion (assuming: 1.2 g rat heart contains 1 ml of water, 0.2 g dry weight). Considering the intracellular H^+ buffering power (β) is within mM range (see 30 for review), it is not surprising to find out the concentration of accumulation of H^+ load is far more higher than pH_i. However, it is uncertain whether the change of accumulated H^+ production is proportionally associated with the change of pH_i. This is because there are a number of factors (such as H^+ extrusion pathways) may change β (see 30 for review).

Effects of palmitate on high energy phosphate

While a high level of fatty acid increased H^+ production in the heart, it is possible that the detrimental effects of fatty acids may be due to alterations in high energy phosphate production. However, in this study, palmitate did not have any significant effects on ATP, PCr or Pi content measured at the end of ischemia, and the content of ATP or Pi during reperfusion (Fig. 4-7) did not predict the extent of recovery. This lack of correlation between actual levels of high energy phosphates and the recovery of mechanical function parallels previously observed[32,33]. In the present study, the recovery of ATP content was poor in all groups while the recovery of PCr content was significantly higher in Glucose and Glucose+Palmitate+DCA groups. A high level of PCr content suggests a high level of ATP resynthesis and indicates better preservation of mitochondrial function. Since better recovery of cardiac function and efficiency were found in Glucose and Glucose+Palmitate+DCA hearts, it is possible that less Na^+ and Ca^{2+} overload may have occurred in the above two groups and less ATP may be consumed for restoration of ion homeostasis. Therefore, even though ATP content did not recover in any of the groups, it appears that the efficiency of utilization of ATP was different.

Relationships between cardiac work, coronary flow and recovery of pH_i

In this study, coronary flow was no difference between Glucose+Palmitate and Glucose group during baseline aerobic perfusion, but was 59% lower in the Glucose+Palmitate group compared with the Glucose group during reperfusion. However, CVR data indicate that the poor coronary perfusion is a consequence of lower workload in the reperfused hearts. In the present study, CVR, a robust measure of coronary vascular tone is not different among Glucose, Glucose+Palmitate, and Glucose+Palmitate+DCA groups (Table 4-1,4-3). The lower coronary flow, in combination with an identical CVR, is due to a lower coronary perfusion pressure in the Glucose+Palmitate hearts. The hydrostatic afterload, against which all hearts had to eject, was fixed at 80 mmHg in all groups during baseline aerobic perfusion. However, during postischemic reperfusion the Glucose+Palmitate hearts has impaired contractility and cardiac output. Some hearts did not eject sufficient perfusate to maintain an aortic flow and a hydrostatic afterload of 80 mmHg. Thus, poor contractile function is the basis for the lower afterload and lower coronary flow. Furthermore, unpublished data from this research group has shown that, in hearts perfused for 80 min under normal aerobic conditions, there is no difference in coronary flow, afterload, and hence coronary perfusion pressure, between Glucose and Glucose+Palmitate groups. This indicates that the presence of palmitate has no significant effect on coronary flow. Therefore, the low coronary flow observed in the Glucose+Palmitate group is not simply due to a coronary occlusive action of palmitate, but appears due to the low level of cardiac function caused by fatty acid induced-H^+ accumulation.

Clinical Implications

Manipulation of energy substrate metabolism is a novel approach to treating ischemic heart disease. Considerable interest has refocused on the use of glucose, insulin, and potassium (GIK) in both acute myocardial ischemia and cardiac surgery patients[34-36]. A recent study by Diaz et al[36] showed that for the treatment of acute myocardial infarction, patients receiving GIK therapy have a statistically significantly reduction in mortality. Although not directly determined, GIK should lower fatty acids levels in the blood[19], which may result in a decreased H^+ production in the heart. This possibility remains to be determined, although clinical studies are presently underway to assess this.

Summary

During reperfusion, a high level of fatty acid delays the recovery of pH_i by increasing intracellular H^+ production from glycolysis uncoupled from glucose metabolism. This impairs the recovery of contractile function and cardiac efficiency in postischemic working rat hearts. Reducing H^+ production by stimulation of glucose oxidation during reperfusion significantly improves the coupling of glucose metabolism, and therefore accelerates pH_i recovery. This leads to a significant improvement of the recovery of cardiac function and efficiency.

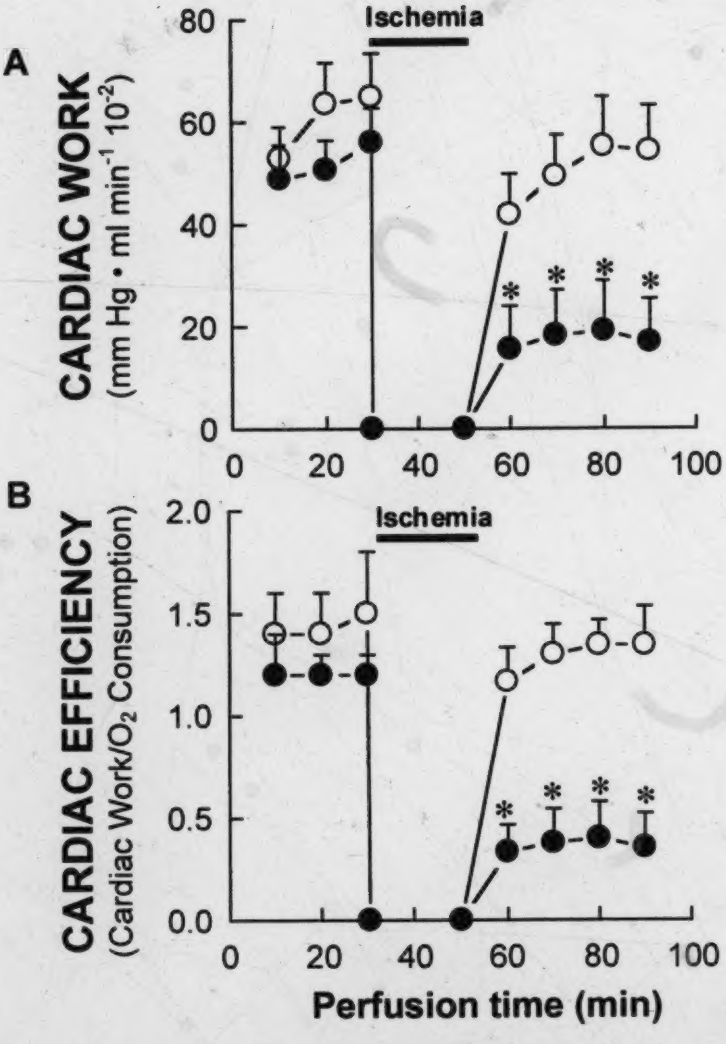

Figure 4-1 Effects of palmitate on the recovery of cardiac work (A) and cardiac efficiency (B) of isolated working rat hearts reperfused after ischemia. Values are mean ± SEM of 8 Glucose hearts (O) and 8 Glucose+Palmitate (●). *, Significantly different from Glucose hearts at the corresponding perfusion time.

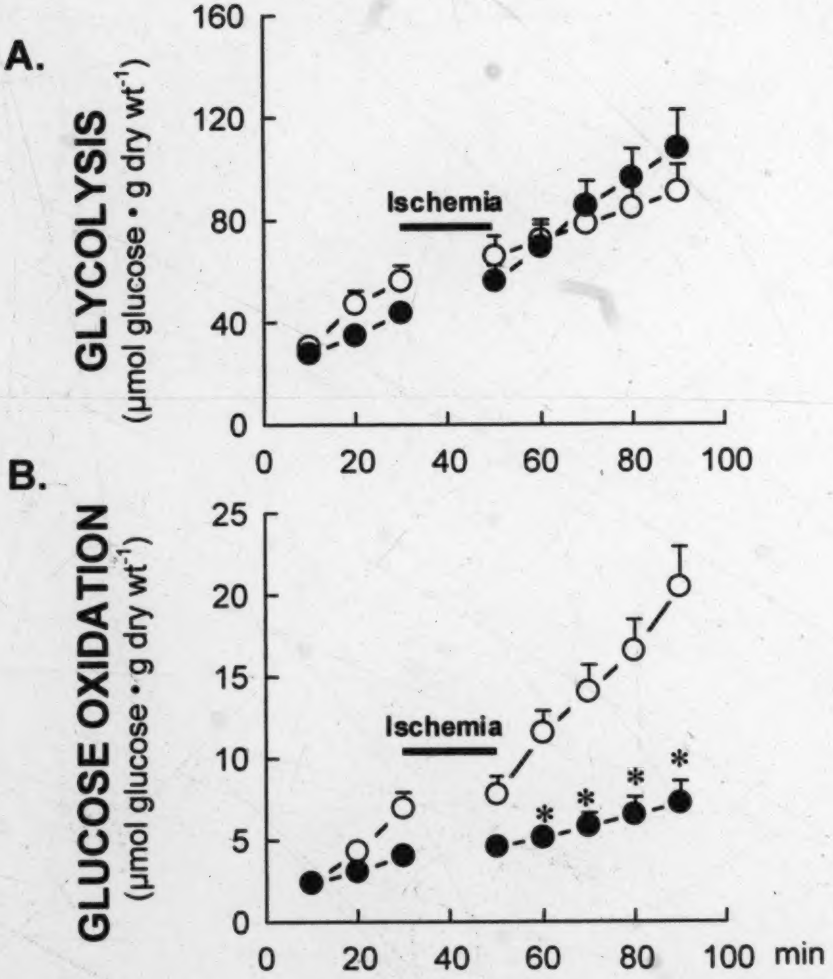

Figure 4-2. Effects of palmitate on the time-course of glycolysis (A) and glucose oxidation (B).

Values are mean ± SEM of 8 Glucose hearts (○) and 8 Glucose+Palmitate hearts (●). *, Significantly different from Glucose hearts at the corresponding perfusion time.

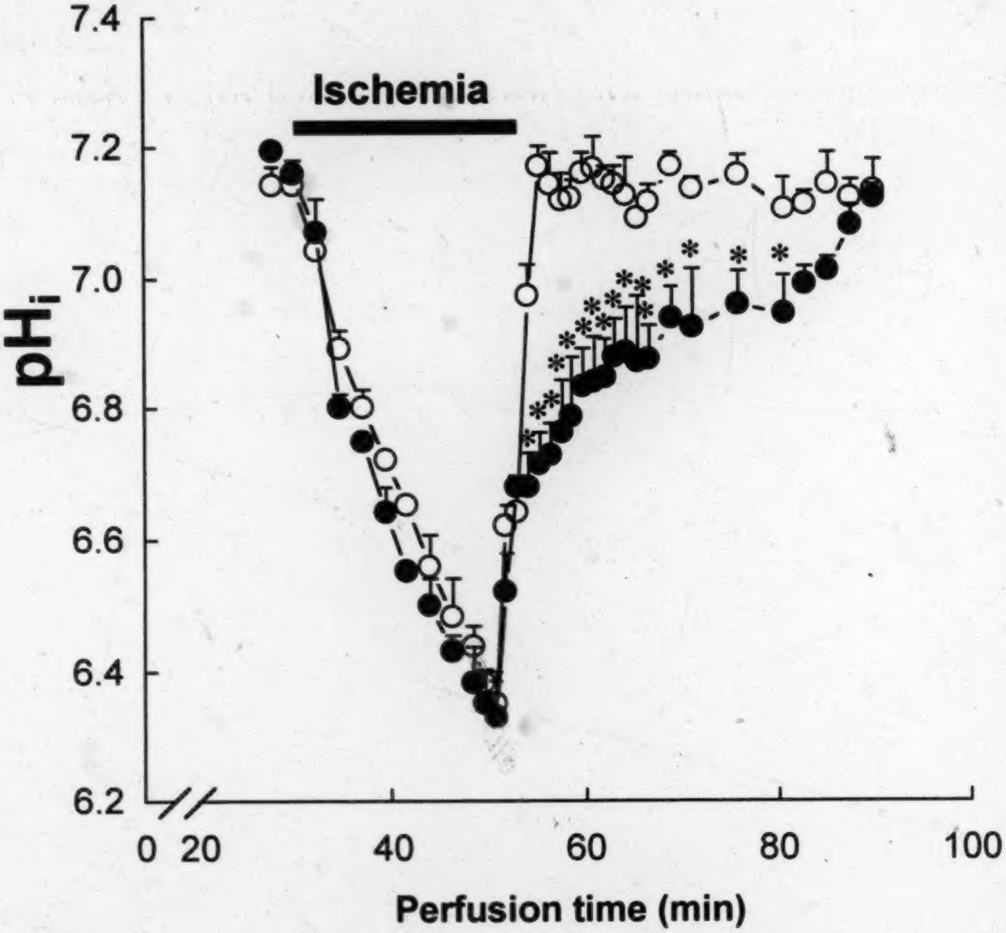

Figure 4-3. Effect of palmitate on pH_i during ischemia and reperfusion in isolated working hearts.

Values are mean ± SEM of 9 Glucose hearts (○) and 9 Glucose+Palmitate hearts (●). *, Significantly different from Glucose hearts at the corresponding perfusion time.

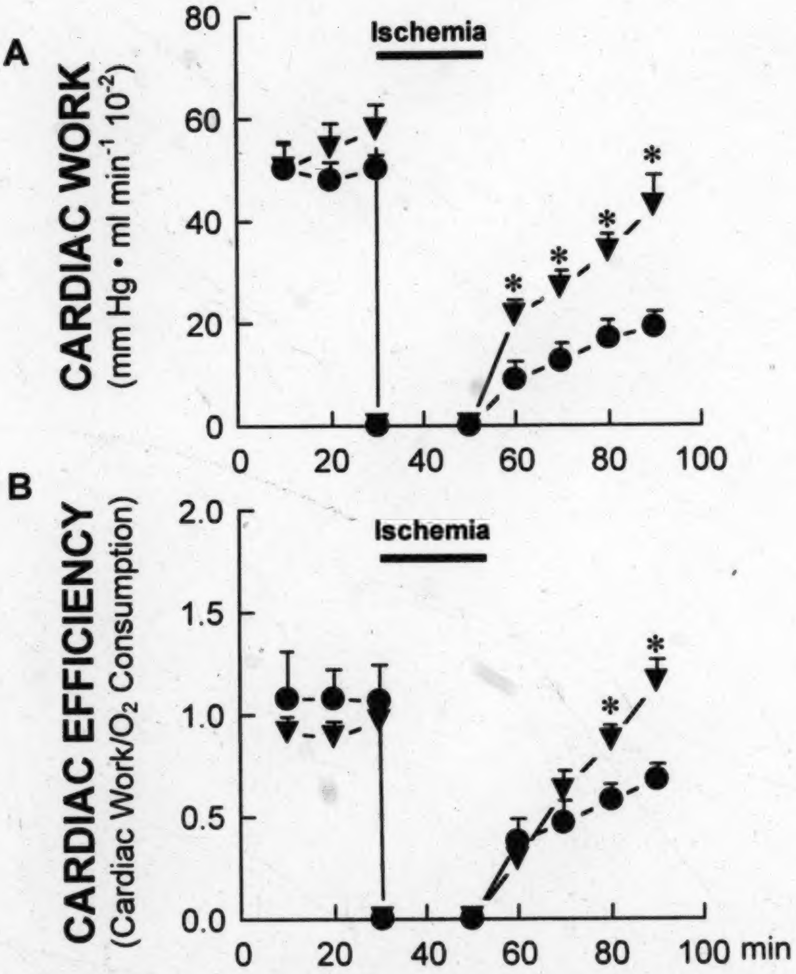

Figure 4-4. Effects of DCA on the recovery of cardiac work (A) and cardiac efficiency (B).

Values are mean ± SEM of 8 Glucose+Palmitate hearts (●) and 8 Glucose+Palmitate+DCA hearts (▼). *, Significantly different from Glucose+Palmitate hearts at the corresponding perfusion time.

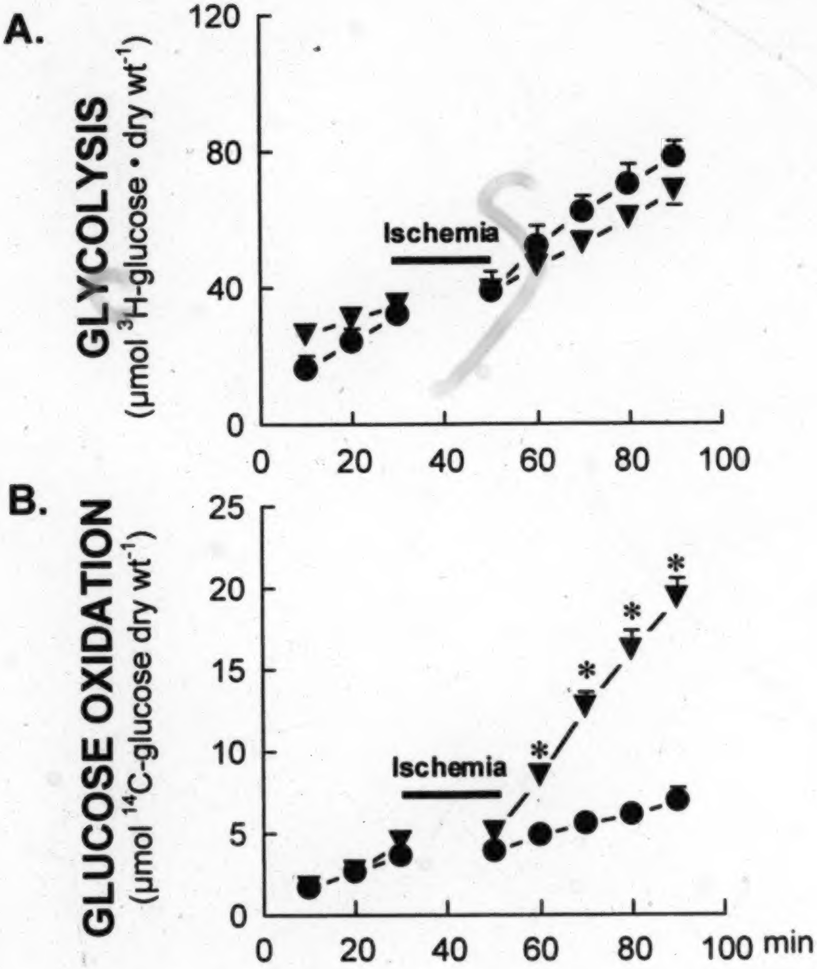

Figure 4-5. Effects of DCA on the time-course of glycolysis (A) and glucose oxidation (B).

Values are mean ± SEM of 8 Glucose+Palmitate-perfused hearts (●) and 8 Glucose+Palmitate+DCA-perfused hearts (▼). *, Significantly different from Glucose+Palmitate-perfused hearts at the corresponding perfusion time.

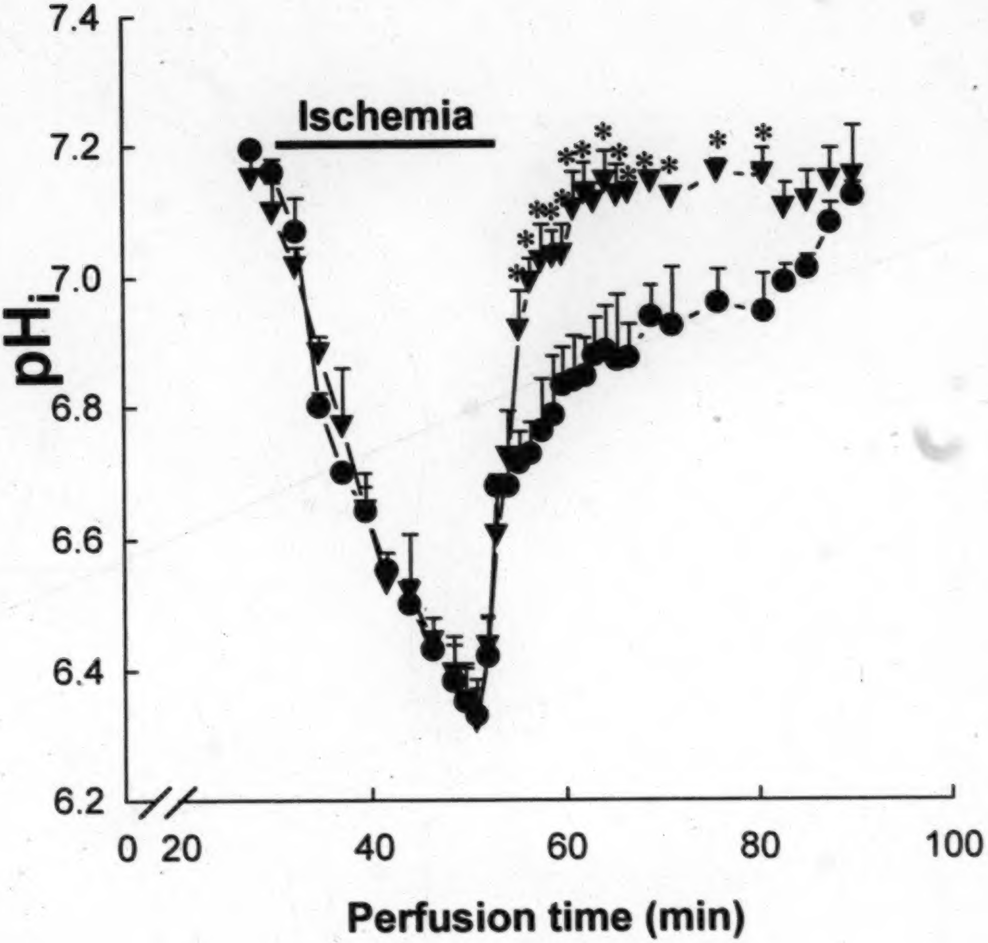

Figure 4-6. Effect of DCA on pH$_i$ during ischemia and reperfusion.

Values are mean ± SEM of 8 Glucose+Palmitate hearts (●) and 8 Glucose+Palmitate+DCA hearts (▼). DCA (3 mM) was added immediately before reperfusion. *, Significant different from Glucose+Palmitate hearts at the corresponding perfusion time.

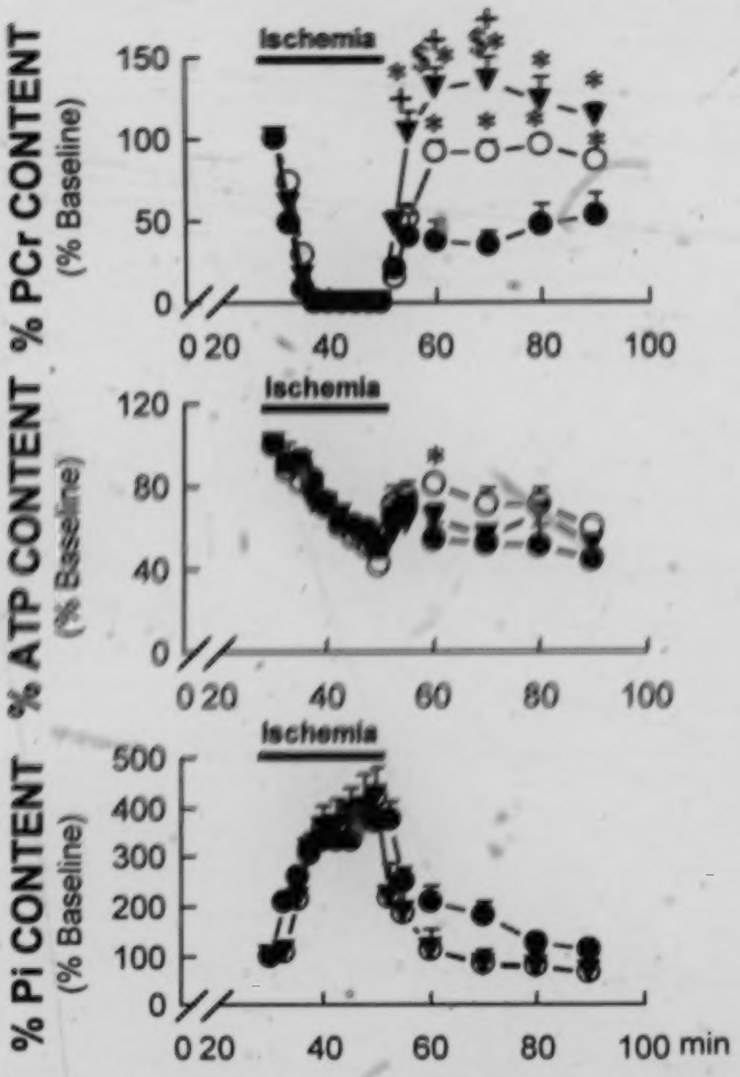

Figure 4-7. PCr, ATP and Pi as percent of baseline values in hearts.

Values are mean ± SEM for 8 Glucose hearts (○), 8 Glucose+Palmitate hearts (●), and 8 Glucose+Palmitate+DCA hearts (▼).

*, Significantly different from Glucose+Palmitate hearts.

+, Significantly different between Glucose+Palmitate+DCA and Glucose hearts.

§, Significantly different from preischemic baseline value.

Table 4-1. Effect of 1.2 mM palmitate on the recovery of mechanical function of isolated working rat hearts subjected to global no-flow ischemia.

Parameters Measured	Glucose (8) Pre/Postischemic		Glucose+Palmitate (8) Pre/Postischemic	
Heart rate, beats • min^{-1}	233±8	[illegible]±2	238±8	105±26*†
Peak systolic pressure, mmHg	130±13	123±17	124±12	68±13*†
Developed pressure, mmHg	70±13	67±15	71±17	23±11*†
Cardiac output, ml • min^{-1}	50±4	44±5	45±4	25±4*†
MVO$_2$, µmol • g dry wt^{-1} • min^{-1}	48±4	45±8	47±5	24±4*†
Coronary flow, ml • min^{-1}	21±2	22±2	21±2	9±4*†
CVR, mmHg • min • ml^{-1}	6.6±1.1	4.4±0.7	6.0±0.8	4.6±0.7

Values are means ± SEM. Hearts were subjected to 30 min of aerobic perfusion, 20 min of global no-flow ischemia, and 40 min of aerobic reperfusion. Preischemic values were taken after 30 min of aerobic perfusion. Postischemic values were taken after 40 min of reperfusion.

*, Significantly different from postischemic values in Glucose hearts.

†, Significantly different from preischemic values

Table 4-2. Effects of 1.2 mM palmitate on rates of glycolysis, glucose oxidation, and H^+ production from glucose metabolism during reperfusion of ischemic hearts.

Parameter Measured (nmol • g dry wt^{-1} • min^{-1})	Glucose (n=8)	Glucose+Palmitate (n=8)
Glycolysis	586±48	637±84
Glucose oxidation	319±49	69±26*
H^+ production	534±47	1136±85*

Values are means ± SEM of the number of hearts indicated. Hearts were subjected to 30 min of aerobic perfusion, 20 min of global no-flow ischemia, and 40 min of aerobic reperfusion. Postischemic values were determined between 10 and 40 min of reperfusion.

*, Significantly different from postischemic values in Glucose hearts.

Table 4-3. Effects of DCA on the recovery of mechanical function of isolated working rat hearts subjected to global no-flow ischemia.

Parameters Measured	Glucose+Palmitate Pre/Postischemic		Glucose+Palmitate+DCA Pre/Postischemic	
Heart rate, beats • min^{-1}	234±7	106±22†	238±8	225±26
Peak systolic pressure, mmHg	120±13	70±12†	128±12	112±13*
Developed pressure, mmHg	71±13	22±12†	71±17	58±11*
Cardiac output, ml • min^{-1}	42±4	26±3†	45±5	39±4*
MVO$_2$, µmol • g dry wt^{-1} • min^{-1}	52±5	27±3†	62±6	36±10†*
Coronary flow, ml • min^{-1}	21±2	16±2†	21±2	16±3*
CVR, mmHg • min • ml^{-1}	5.8±0.5	4.6±0.6	6.0±0.2	6.1±0.6

Values are means ± SEM of the number of hearts indicated. Hearts were subjected to 30 min of aerobic perfusion, 20 min of global no-flow ischemia, and 40 min of aerobic reperfusion. Preischemic values were taken after 30 min of aerobic perfusion. Postischemic values were taken after 40 min of reperfusion. DCA (3 mM), when present, was added immediately before reperfusion.

*,Significantly different from postischemic values in Glucose+Palmitate hearts.

†,Significantly different from preischemic values.

Table 4-4. Effects of DCA on rates of glycolysis, glucose oxidation, and H$^+$ production from glucose metabolism during reperfusion of ischemic hearts perfused with glucose+palmitate.

Parameter Measured (nmol • g dry wt^{-1}• min^{-1})	Glucose+Palmiate (n=8)	Glucose+Palmitate+DCA (n=8)
Glycolysis	678±100	743±66
Glucose oxidation	96±16	356±31*
H$^+$ production	1164±66	770±65*

Values are means ± SEM of the number of hearts indicated. Hearts were subjected to 30 min of aerobic perfusion, 20 min of global no-flow ischemia, and 40 min of aerobic reperfusion. Postischemic values were determined between 10 and 40 min of reperfusion. DCA (3 mM), when present, was added immediately before reperfusion.

*, Significantly different from postischemic values in Glucose+Palmitate hearts.

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Chapter 5

Cardiac efficiency in postischemic hearts is improved by either inhibiting Na^+/H^+ exchange or reducing intracellular H^+ production

1. Introduction

During ischemia, oxidative metabolism is suppressed and anaerobic glycolysis becomes an important source of ATP production[1]. H^+ production from the hydrolysis of glycolytically-derived ATP is a major contributor to the acidosis that occurs in the ischemic myocardium[2]. Upon reperfusion, extracellular pH quickly recovers. This creates large pH gradients across the membrane and NHE1 is activated[3,4]. The subsequent increase in intracellular Na^+ may exchange with Ca^{2+} via the Na^+/Ca^{2+} exchanger (NCE) leading to intracellular Ca^{2+} overload and cell death[5,6]. A number of studies have demonstrated that NHE1 inhibitors improve the recovery of cardiac function and attenuate intracellular Ca^{2+} overload during reperfusion[7-9]. Therefore, accumulation of intracellular H^+ during ischemia is an important contributing factor to ischemia/reperfusion injury. As discussed in the previous Chapter, continued production of H^+ during the critical early period of reperfusion also have the potential to exacerbate this injury[10,11].

In most clinical situations of reperfusion, the heart muscle is exposed to high levels of fatty acids[12]. Our previous studies have shown that high rates of fatty acid oxidation inhibit glucose oxidation, which results in a marked imbalance between rates of glycolysis and glucose oxidation[11,13,14]. This is associated with a poor recovery of cardiac function and efficiency. This uncoupling of glucose metabolism is a major source of H^+ production for intracellular acidosis during ischemia and reperfusion[10,13,14]. If glycolysis is coupled to glucose oxidation, H^+ production from glucose metabolism is zero[10,15]. However, if glycolysis is uncoupled from glucose oxidation (and pyruvate derived from glycolysis is not oxidized), the hydrolysis of glycolytically derived ATP results in a net production of $2H^+$ for each glucose molecule. Data in Chapter 4 showed that in the presence of a high level of fatty acid, an increased H^+ production generated from uncoupled glucose metabolism occurs, which significantly delays the recovery of pH_i during reperfusion[16] (see chapter 4 for details). Reduction of H^+ production by stimulating glucose oxidation accelerates the recovery of pH_i and improves the recovery of cardiac function and efficiency after ischemia[16]. Thus, besides intracellular acidosis generated during ischemia, the continually generated H^+ production during reperfusion may also contribute to the reperfusion injury via activation of NHE1. In the present study, we further investigated, in the presence of a high level of fatty acid which generates H^+ load, the effects of inhibiting NHE1 and reducing the rate of H^+ production on the recovery of pH_i and cardiac efficiency during reperfusion in postischemic working hearts. We proposed that the continually generated H^+ production during reperfusion plays an important role in the activation of NHE1 and is detrimental for the recovery of cardiac function and efficiency.

2. Methods (For general experimental methods, please see Chapter 2)

Glycolysis and glucose oxidation rates were measured simultaneously by perfusing hearts with [5-^{3}H/U-^{14}C]. Overall rates of H^+ production derived from glucose metabolism were determined by subtracting the rate of glucose oxidation from the rate of glycolysis and multiplying by two. pH_i and high energy phosphate were measured by ^{31}P-NMR.

3. Experimental Protocol

Isolated working rat hearts were subjected to 30 min of aerobic perfusion, 20 min of global no-flow ischemia (33 °C) and 50 min of reperfusion. CAR (cariporide) was added 5 min before ischemia at a final concentration of 5 μM. DCA was added immediately before reperfusion of ischemic hearts at a 3 mM final concentration. Hearts were divided into 4 groups: 1) Control (5.5 mM glucose + 1.2 mM palmitate), n=8; 2) CAR (5.5 mM glucose + 1.2 mM palmitate + CAR 5 μM), n=8; 3) DCA (5.5 mM glucose + 1.2 mM palmitate + DCA 3 mM), n=8; 4) CAR+DCA (5.5 mM glucose + 1.2 mM palmitate + CAR 5 μM + DCA 3 mM), n=8. Hearts were perfused with krebs-Henseleit solution containing 5.5 mM glucose, 1.2 mM palmitate.

4. Results

4.1 Effects of CAR and DCA on the recovery of cardiac function and efficiency

Fig 5-1A shows the effects of 5 μM CAR and 3 mM DCA on the recovery of cardiac function and efficiency in hearts subjected to 20 min of global ischemia. After ischemia, the recovery of cardiac work was depressed in control hearts, returning to only 38±6% of the preischemic value at 50 min of reperfusion. In DCA-treated hearts, cardiac work recovered to 80±6% of preischemic levels ($p<0.05$ compared with recovery in control). Although in CAR- or CAR+DCA-treated hearts, a complete recovery of cardiac work was seen, this recovery was slower than DCA-treated hearts. For instance, the recovery of cardiac work in CAR- or CAR+DCA-treated hearts was not significantly improved in the first 30 min of reperfusion, while the recovery of cardiac work in DCA-treated hearts was significantly greater than control during the first 30 min of reperfusion($p<0.05$).

MVO_2 in CAR-, DCA- or CAR+DCA-treated hearts did not recover to the same extent as cardiac work (Table 5-1), resulting in a dramatic increase in cardiac efficiency at the end of reperfusion (Fig. 5-1B). Therefore, treatment with CAR, DCA or CAR+DCA significantly improved the recovery of cardiac function and efficiency during reperfusion.

4.2 Effects of CAR and DCA on glucose metabolism and calculated H^+ production

Fig 5-2 shows the amount of substrate metabolized versus time for glycolysis (panel A) or glucose oxidation (pane B). The effects of CAR, DCA and CAR+DCA on steady-state rates of glycolysis, glucose oxidation and calculated H^+ production are shown in Table 5-2. There was no significant difference in glycolysis among control, CAR-, DCA-, and CAR+DCA-treated hearts (Fig. 5-2A). Although treatment with CAR did not significantly affect glucose oxidation rate (Table 5-2), cumulative glucose oxidation was increased compared with control ($p<0.05$) at the end of reperfusion period (Fig. 5-2B). This is possibly because glucose metabolism is closely associated with the status of cardiac work. In this study, cardiac work completely recovered at the end of reperfusion in the CAR-treated hearts.

Glucose oxidation rate was significantly increased in DCA- or CAR+DCA-treated hearts during reperfusion. This led to a significant increase in the rate of glucose oxidation and a significant reduction in H^+ production rate calculated from glucose metabolism during the 50 min of reperfusion (Table 5-2). Since the steady-state rate of either glycolysis or glucose oxidation was not significantly increased in CAR-treated hearts, the calculated H^+ production rate was not significantly altered during reperfusion.

4.3 Effects of CAR and DCA on the recovery of pH_i and high energy phosphates

The effects of CAR and DCA on pH_i during the ischemia and reperfusion periods are shown in Fig. 5-3. A greater acidosis occurred in CAR and CAR+DCA groups compared with control during ischemia. Since DCA was added immediately before reperfusion, there was no difference in pH_i during ischemia between CAR and CAR+DCA groups. During reperfusion, treatment with CAR or CAR+DCA significantly delayed the recovery of pH_i compared with control hearts. However, although rates of recovery of pH_i were delayed, pH_i did recover to preischemic level at the end of reperfusion in control, CAR and CAR+DCA groups. In contrast, pH_i quickly recovered to preischemic level within 10 min of reperfusion in DCA-treated hearts. Although there was no difference in the rate of recovery of pH_i among control, CAR and CAR+DCA groups (0.022±0.003, 0.017±0.003, 0.023±0.004 pH unit • min^{-1}, respectively) during the first 30 min of reperfusion, the absolute pH_i value in the CAR group was significantly lower than in control group. During the last 20 min of reperfusion, the rate of recovery of pH_i in CAR-treated hearts was faster than control and CAR+DCA groups (0.021±0.003 vs 0.004±0.0005, 0.008±0.003 pH unit • min^{-1}, $p<0.05$, respectively). Although within the first 10 min of reperfusion, there was no difference in pH_i between the CAR and CAR+DCA groups, the rate of recovery of pH_i was faster in the CAR+DCA group between 10 and 30 min (60 to 80 min) of reperfusion compared with the CAR group (0.014±0.002 vs 0.005±0.0006 pH unit • min^{-1}, $p<0.05$). This may be due to the increased glucose oxidation rate and reduced H^+ production observed in the CAR+DCA-treated hearts.

The changes of high energy pho[illegible] DCA and CAR+DCA groups during ische[illegible] 5-4. During ischemia, PCr content qui[illegible] among the 4 groups. During reperfusion, PCr content in the control hearts only partially recovered. In CAR-, DCA- and CAR+DCA-treated hearts, PCr content completely recovered during reperfusion and was higher than preischemic levels (p>0.05). The recovery of PCr content in DCA treated hearts was the fastest among all the 4 groups (p<0.05). Compared with control and DCA groups, ATP content was significantly lower in CAR-treated hearts during the first 10 min of reperfusion. At the end of reperfusion, there was no difference in ATP content among all groups and in contrast to the recovery of PCr content, ATP content did not completely recover. There was no significant difference in inorganic phosphate (Pi) content among these 4 groups at any time point during ischemia or during reperfusion.

5. Discussion

We demonstrated that in the pre[illegible] inhibition of NHE1 with cariporide (CAR, 5 μM) improved the recovery of cardiac function and efficiency although this was associated with a significant slower recovery of pH_i during reperfusion. Improving the coupling of glycolysis to glucose oxidation by stimulation of glucose oxidation with dichloroacetate (DCA, [illegible] further improvement in cardiac efficiency during reperfusion. Our results suggest that it is not the rate of recovery of pHi, per se but rather the source and the fate of H^+ that is important for the recovery of cardiac function and efficiency during reperfusion.

An attractive mechanism proposed for ischemia-reperfusion injury implicates intracellular acidosis as a trigger[illegible]. Previous studies have suggested that H^+ production generated from uncoupled glucose metabolism stimulates NHE1, adding to Na^+ influx[illegible]. Increased intracellular Na^+ load can subsequently cause intracellular Ca^{2+} overload via NCE[illegible]. A number of studies have demonstrated that NHE1 plays an important role in the [illegible]

Although intracellular acidosis generated during ischemia seems to play an important role in the pathogenesis of ischemia-reperfusion injury, several studies offer evidence to dissociate ischemic acidosis from postischemic [illegible], although they significantly attenuate [illegible] of postischemic hearts. This was also observed in our study in which [illegible] improved functional recovery of postischemic hearts, although the recovery of pHi was significantly slower during reperfusion and pHi was significantly decreased during ischemia compared to control hearts. Thus, intracellular acidosis itself may be a lesser problem compared to the consequences of NHE1-driven Na^+ entry during ischemia and reperfusion. Furthermore, upon reperfusion, NHE1 activity is stimulated due to rapid normalization of extracellular pH and the generation of H^+ gradient[illegible]. A number of studies[illegible] have demonstrated that Na^+ and Ca^{2+} overload occurs in the early period of reperfusion and plays an important role in the ischemia-reperfusion injury.

[illegible] reduce H^+ gradient and result in a lesser activation of NHE1, subsequently resulting in improvement of cardiac function and efficiency. Our previous studies (see Chapter 4 for details) have suggested that in the presence of high levels of fatty acids, H^+ production generated from uncoupled glucose metabolism during reperfusion is an important contributor to the impaired recovery of mechanical [illegible] [illegible] [illegible] efficiency (see also Chapter 4 of this thesis). Reducing H+ production by stimulating glucose oxidation accelerated the rate of recovery of pH, and improved the recovery of cardiac function and efficiency. In the present study, we addressed the issue that whether altering both the rate and source of H+ production can affect the recovery of cardiac efficiency during reperfusion. We also determined whether the recovery of pH, per se during reperfusion is important to the recovery of cardiac efficiency.

In this study, treatment with either CAR or DCA improved the recovery of cardiac function and efficiency in postischemic isolated working rat hearts subjected to 20 min of global ischemia (Fig. 5-1). Treatment with DCA (1-5 mM) has been shown by a number of investigators to improve the functional recovery of [illegible] [illegible] [illegible] production, a more rapid recovery of pH, and a significant improvement in cardiac efficiency[16]. Treatment with DCA during reperfusion does not affect glycolysis and fatty acid oxidation[11,13]. To our knowledge, the beneficial effect of DCA on the recovery of cardiac function and efficiency is mainly due to activation of PDH. In this study, glucose oxidation rate was 6.9-fold lower than glycolytic rate in control hearts (Table 5-2). In contrast, glucose oxidation was significantly increased by 2.2-fold in DCA-treated hearts during reperfusion compared with control (Fig.5-2). Glucose oxidation rate was 56±5% of glycolytic rate in DCA-treated hearts. This uncoupled glucose metabolism in control hearts generated a substantial H^+ load during the critical period of reperfusion compared with DCA-treated hearts (1184±89 vs 564±65 nmol • g dry wt^{-1} • min^{-1}, $p<0.05$). In CAR-treated hearts, glucose metabolism and H^+ production were not significantly changed compared with control. Thus, the recoveries of pH_i in control hearts and in CAR-treated hearts were significantly slower than in DCA-treated hearts (Fig. 5-3).

Although NHE1 plays an important role in the regulation of pH_i[7-9], it is uncertain whether inhibiting NHE1 during ischemia accentuates intracellular acidosis (see 29 for review). In rat cardiac myocytes CAR inhibited pH_i recovery after intracellular acidification with an IC_{50} between 1 and 0.1 μM[30]. A recent study has also shown that CAR (1 μM) does not affect pH_i during ischemia in isolated rat hearts[31]. In this study, we first showed that pH_i decreased by a greater extent in CAR-treated hearts (5 μM) during ischemia. Of interest is that although the pH_i values in CAR group were significantly lower than in control, there was no difference in the rates of recovery of pH_i between control and CAR groups during the initial 30 min of reperfusion. However, for the last 20 min of reperfusion, the rate of recovery of pH_i in CAR group was significant faster than in the control group. Since there are at least four mechanisms responsible for the compensation of myocardial acidosis during reperfusion:NHE1[7], lactate-H^+ co-transporter (MCT)[32], vacuolar–H^+ ATPase[33], and Na^+/HCO_3^{-1} cotransporter[34], at least one of the other H^+ extrusion pathways may be hyperactivated during the last 20 min of reperfusion, which caused a quick recovery of pH_i in the CAR-treated group. However, it is not clear why the rate of recovery of pH_i in CAR-treated hearts is similar to the control hearts during the initial 30 min of reperfusion, although the absolute pH_i value in the CAR group was significantly lower in the control group. Despite the slow recovery of pH_i, cardiac function and efficiency in CAR-treated hearts recovered completely by the end of reperfusion. Of interest is that the slow recovery of pH_i in the CAR-treated hearts was associated with the depressed cardiac function and efficiency during early period of reperfusion. This may be because intracellular acidosis induced by inhibition of NHE1 inhibits cardiac contractile function[35,36].

Treatment with DCA led to a faster recovery of pH_i and better recovery of mechanical function and efficiency compared with control. This fits with results of our previous studies (see Chapter 4 for details). However, in CAR-treated hearts, a slower recovery of pH_i was associated with a complete recovery of cardiac function and efficiency. These data indicate that: 1) the recovery of pH_i *per se* is not important for the recovery of cardiac function and efficiency; 2) inhibition of NHE1, despite slowing the recovery of pH_i during reperfusion, is an important factor for the improvement of cardiac function and efficiency; 3) H^+ production generated during reperfusion activates NHE1 and may drive Na^+ and

Ca^{2+} influx. Reducing H^+ production can reduce NHE1 activation and improve the recovery of cardiac function and efficiency. Therefore, it is the amount of intracellular H^+ load cleared by the NHE1 that is important for the depressed recovery of cardiac function and efficiency. Either reducing H^+ production with DCA or inhibition of NHE1 with CAR may attenuate intracellular Na^+ and Ca^{2+} overload and improve the recovery of cardiac function and efficiency.

The slower recovery of pH_i observed in the CAR-treated hearts compared to previous studies[26,31] may be due to 1) the presence of a high level of fatty acid, which is the major fuel for the heart and generates H^+ production by inhibiting glucose oxidation; or 2) a working heart model, which was employed to measure pH_i instead of isolated cells or Langendorff perfused (non working) hearts (see Chapter 1 &7 for details).

In the present study, we found that, in CAR+DCA-treated hearts, the rate of recovery of pH_i was significantly accelerated compared with CAR-treated hearts (Fig. 5-3). This was due to a significant decrease in H^+ production calculated from glucose metabolism in CAR+DCA-treated hearts since glucose oxidation was significantly increased during reperfusion (Fig.5-2, Table 5-2). Since cardiac function and efficiency completely recovered at the end of reperfusion in both CAR and CAR+DCA-treated groups (Fig.5-1) we could not determine whether the combination of CAR with DCA had any synergistic effect. Similar to the CAR-treated hearts, the recovery of cardiac function and efficiency in CAR+DCA-treated hearts was poor during the initial 30 min of reperfusion. This could reflect the fact that under a low pH_i condition due to inhibition of NHE1, treatment with DCA during the early period of reperfusion could not effectively activate PDH, stimulate glucose oxidation or reduce H^+ production. This is supported by the observation that during the initial 30 min period of reperfusion no difference in glucose oxidation was observed between control and CAR+DCA-treated hearts (Fig. 5-2). Although at 30 min of reperfusion, the recovery of pH_i in the CAR+DCA group was significantly higher than the CAR group (6.95±0.09 vs 6.70±0.06 unit, $p<0.05$), no significant changes in cardiac function and efficiency were found. This may be because low pH_i can inhibit contractile function[3,17]. In contrast, the rapid recovery of pH_i in the DCA group was associated with a rapid recovery of cardiac function and efficiency. In a future study, we will optimize the concentration of CAR and administer CAR only during reperfusion. This may be a better protocol to investigate the combined effects of CAR+DCA on the recovery of pH_i and cardiac efficiency.

It is important to recognize that pH_i, ATP and Pi content measured before reperfusion do not predict the extent of recovery[24], nor does ATP content correlate with contractile function during reperfusion[37]. In this study, ATP contents in the CAR+DCA and CAR-treated hearts were not higher than in control hearts during reperfusion. However, ATP content in the CAR-treated hearts was lower than control hearts during the first 10 min of reperfusion. This may be due to a relatively lower pH_i in CAR-treated hearts. In contrast, PCr contents in DCA-, CAR+DCA- and CAR-treated hearts were higher than in control hearts during reperfusion. At the end of reperfusion, PCr contents in CAR- or CAR+DCA-treated hearts were also higher than preischemic levels. The mechanisms of the improved recovery of PCr contents in CAR- or CAR+DCA-treated hearts are not clear. In the present study, cardiac efficiencies in CAR-, DCA- and CAR+DCA-groups were significantly improved while ATP contents were not significantly different from control hearts. This may indicate an increased efficiency in the utilization of ATP. Therefore, even though the ATP level did not recover, the efficiency of utilization of ATP may be different. As the intracellular Na^+ and Ca^{2+} overload after activation of the NHE1 and NCE requires ATP to restore ion homeostasis[3,4], either inhibition of NHE1 or reducing H^+ production may lead to a greater efficiency in the utilization of ATP. The high level of PCr content observed in CAR, DCA and CAR+DCA groups suggests high levels of ATP resynthesis and indicates better recovery of mitochondrial function[3,4]. Worth mentioning, the high level of PCr and low level of ATP content observed in CAR-, DCA- and CAR+DCA-treated hearts during reperfusion are consistent with previous studies in which NHE1 is inhibited in the postischemic hearts[3,4,38].

MVO_2 in postischemic hearts is relatively high compared with the depressed cardiac function[11,13], an observation observed in this study. The mechanism of this "oxygen paradox" could occur at different levels: basal metabolism, excitation-contraction coupling, and energy production. However, basal MVO_2 in postischemic hearts is not elevated[39]. Thus, this O_2-consuming portion of total MVO_2 may be not responsible for the cardiac inefficiency in postischemic hearts. The fraction of MVO_2 attributable to excitation-contraction coupling is disproportionately high in stunned myocardium and, in fact, impairment of Ca^{2+} handling appears to be responsible for decreased cardiac efficiency in the postischemic hearts (see 40,41 for review). Recently, the abnormality of total Ca^{2+} handling in the postischemic hearts has been characterized[41]. The abnormality consists of a decreased internal Ca^{2+} recirculation, some futile Ca^{2+} cycling, and a decreased Ca^{2+} reactivity of contractility. These changes can account for the energy-wasteful total Ca^{2+} handling and high MVO_2 in postischemic hearts[42]. A report of Hata et al[43] has shown that activation of NHE1 decreases MVO_2 and depresses myocardial contractility and cardiac efficiency. Thus, inhibition of NHE1 or less activation of NHE1 by treatment with DCA may lead to less MVO_2 consumed for heart muscle contractility. The total MVO_2 may not necessary recover to preischemic level but more MVO_2 contributes to the synthesis of ATP or PCr.

In summary, in the presence of a high level of fatty acids which leads to the generation of an increased H^+ load, inhibition of NHE1 dramatically improves the recovery of cardiac function and efficiency during reperfusion. The beneficial effect of NHE1 inhibition is accompanied by a slower recovery of pH_i. Reducing H^+ production by stimulation of glucose oxidation with DCA during reperfusion can also significantly improve the recovery of cardiac function and efficiency, but in contrast to NHE1 inhibition, an accelerated pH_i recovery occurs during reperfusion. This is because the decrease in H^+ production may attenuate Na^+ and Ca^{2+} influx by lessening the activation of NHE1. Combination of DCA with CAR did not show a synergistic effect on the recovery of cardiac function and efficiency. Thus, it is the fate and source of H^+ instead of the recovery of pH_i *per se* that is important for the recovery of cardiac efficiency during reperfusion.

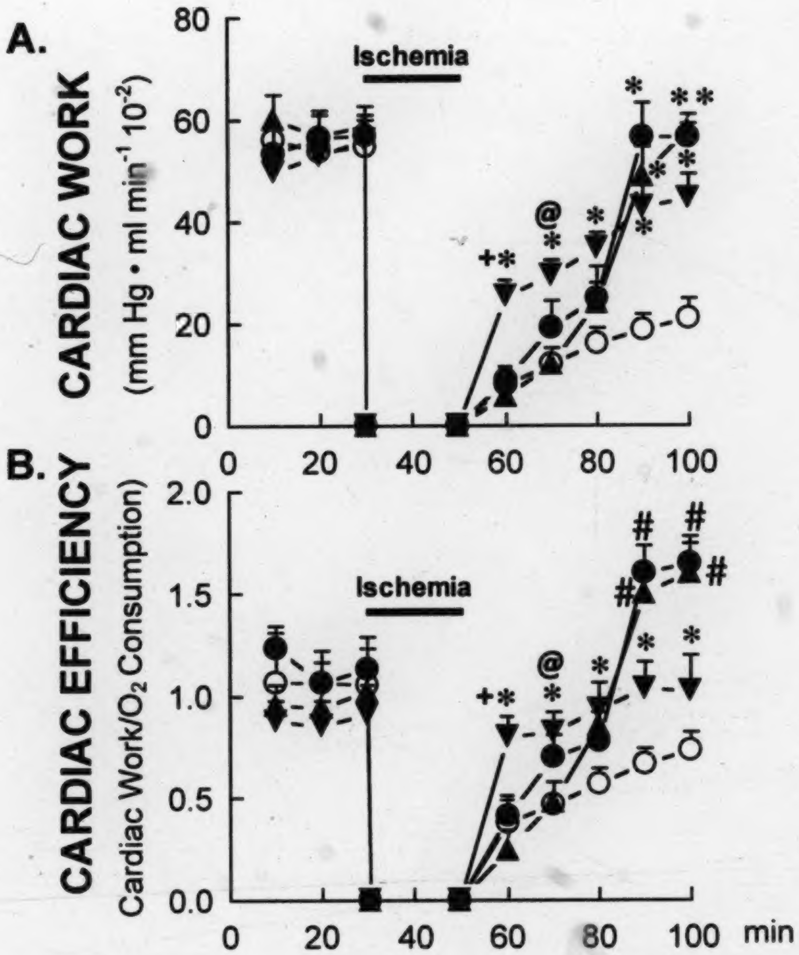

Figure 5-1. Effects of CAR and DCA on the recovery of cardiac work (A) and cardiac efficiency (B) of isolated working hearts reperfused after global no-flow ischemia. Values are mean±SEM. Control (O), n=11;CAR (●), n=8; DCA (▼), n=8; CAR+DCA (▲), n=8. *, Significantly different from control; $^+$, Significantly different between DCA and CAR/CAR+DCA; $^@$, Significantly different between DCA and CAR+DCA; $^\#$, Significantly different between CAR/CAR+DCA and control/ DCA, respectively.

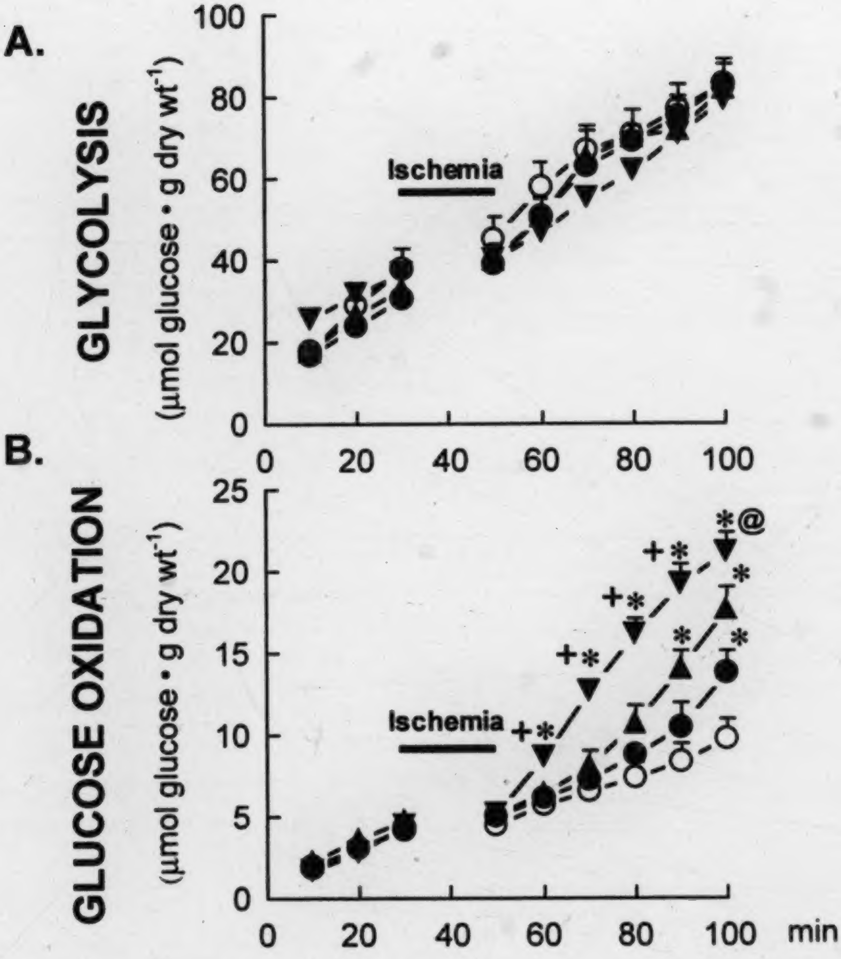

Figure 5-2. Effects of CAR and DCA on the time-course of glycolysis (A) and glucose oxidation (B). Values are mean±SEM. Control (O), n=8; CAR (●), n=8; DCA (▼), n=8; CAR + DCA (▲), n=8. *, Significantly different from control @, Significantly different between DCA and CAR; +, Significantly different between DCA and CAR / CAR +DCA, respectively.

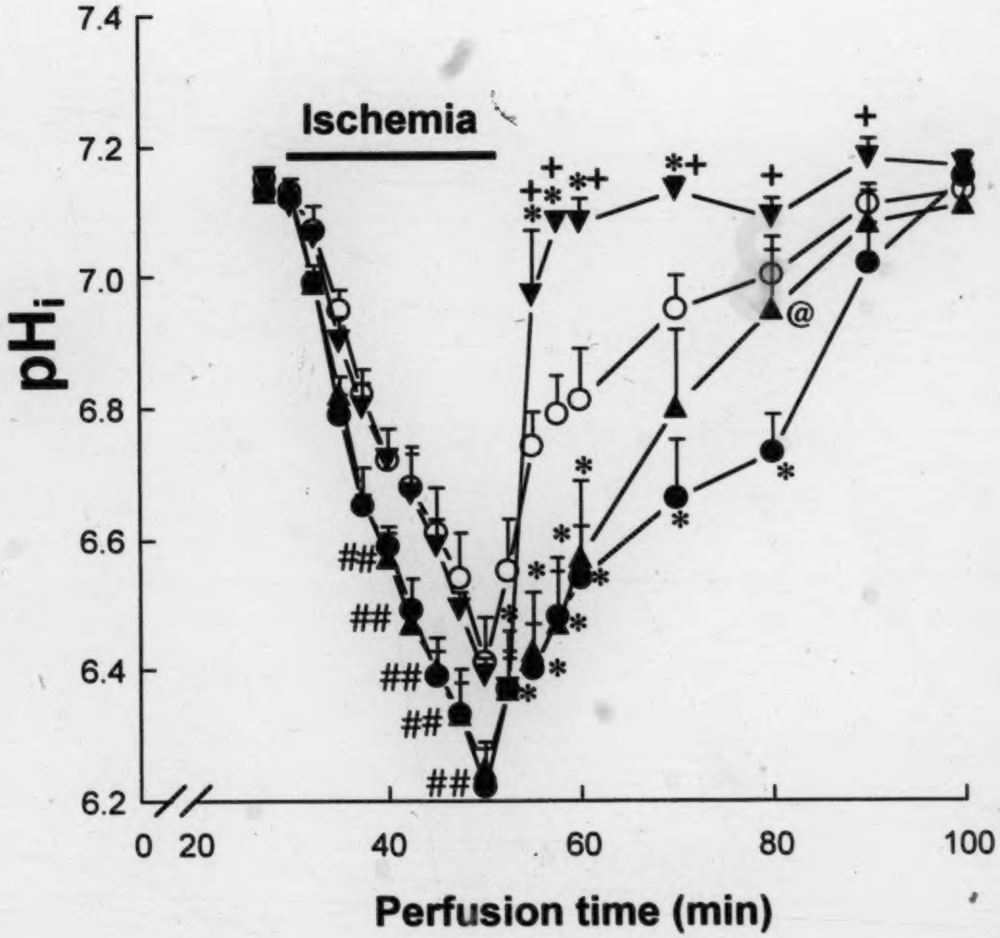

Figure 5-3. Effects of CAR and DCA on pH_i during ischemia and reperfusion. Values are mean±SEM. Control (O), n=11; CAR (●), n=8; DCA (▼), n=8; CAR+DCA (▲), n=8. *, Significantly different from control; $^\#$, Significantly different between CAR/CAR+DCA and DCA/control, respectively; $^@$, Significantly different between CAR+DCA and CAR; $^+$, Significantly different from CAR+DCA and CAR.

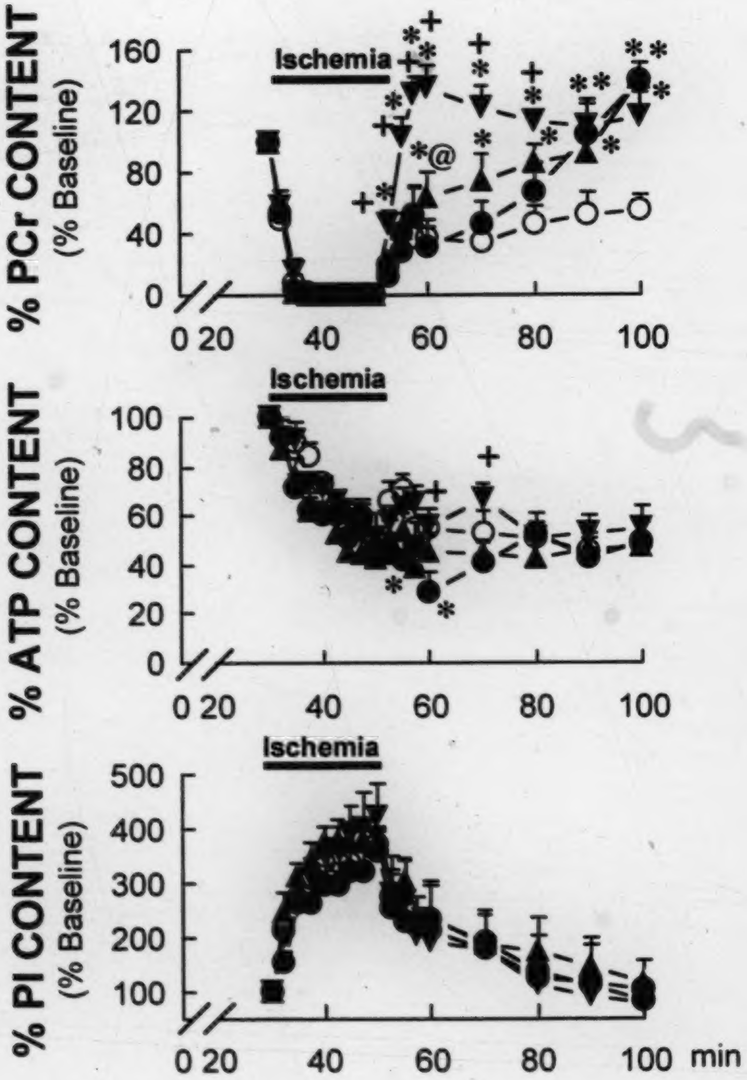

Figure 5-4. PCr, ATP and Pi as percent of baseline values versus time for 11 control (O), 8 CAR (●), 8 DCA (▼), 8 CAR+DCA (▲) in ischemic and postischemic hearts. *, Significantly different from control; $^+$, Significantly different between DCA and CAR/CAR+DCA; $^@$, Significantly different between CAR+DCA and CAR.

Table 5-1. Effects of CAR and DCA on the recovery of mechanical function following ischemia of isolated working rat hearts.

Parameter measured	Control (n=11)	CAR (n=8)	DCA (n=8)	CAR+DCA (n=8)
Preischemic				
Heart rate, beats • min^{-1}	248±8	238±19	235±10	256±17
Peak systolic pressure, mmHg	122±12	125±17	130±12	123±18
Developed pressure, mmHg	71±13	75±8	72±6	75±10
Cardiac output, ml •min^{-1}	45±3	45±4	43±7	48±5
MVO$_2$, μmol • g dry wt^{-1} • min^{-1}	52±5	50±5	62±2	58±5
Coronary flow, ml • min^{-1}	21±2	20±3	22±7	20±4
Postischemic				
Heart rate, beats • min^{-1}	100±22†	201±23*	212±18*	229±26*
Peak systolic pressure, mmHg	70±12†	120±12*	100±12*	122±18*
Developed pressure, mmHg	22±12†	74±12*	71±17*	58±11*
Cardiac output, ml • min^{-1}	28±3†	48±3*	45±5*	48±4*
MVO$_2$, μmol • g dry wt^{-1} • min^{-1}	28±3†	34±5†*	44±5†*	36±3†*
Coronary flow, ml • min^{-1}	7±2†	20±2*	21±2*	19±3*

Values are means ± SEM. Preischemic values were taken after 30 min of aerobic perfusion. Postischemic values were taken after 50 min of reperfusion.

*,Significantly different from postischemic values in control hearts; †, Significantly different from preischemic values.

Table 5-2. Effects of CAR and DCA on rates of glycolysis, glucose oxidation, and H^+ production from glucose metabolism during reperfusion of ischemic hearts.

Parameter Measured (nmol • g dry wt^{-1}• min^{-1})	Control (n=11)	CAR (n=8)	DCA (n=8)	CAR+DCA (n=8)
Glycolysis	688±82	642±72	640±66	624±81
Glucose Oxidation	93±15	168±42	358±31^{*+}	215±25*
H^+ Production	1190±73	958±73	564±65^{*+}	818±82*

Values are means ± SEM of the number of hearts indicated. Hearts were subjected to 30 min of aerobic perfusion, 20 min of global no-flow ischemia, and 50 min of aerobic reperfusion. Values were determined between 10 and 50 min of reperfusion.

*, Significantly different from postischemic values in control group.

$^{+}$, Significantly different from postischemic values in control, CAR and CAR+DCA groups.

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Chapter 6

Effects of H_2O_2 on energy metabolism and pH_i in isolated working rat hearts

1. Introduction

It is well known that myocardial ischemia and reperfusion imposes an oxidant burden on myocardial tissue (see reference 1 for review). The two-electron reduction product of molecular oxygen, hydrogen peroxide (H_2O_2), is an important mediator of cardiac ischemia-reperfusion injury[1,2]. Although a number of studies have demonstrated that H_2O_2 overload inhibits cardiac function, the effects of H_2O_2 on cardiac energy metabolism (fatty acid oxidation, glycolysis and glucose oxidation) are still unknown.

Recent studies have shown that under normal aerobic conditions, acute treatment with H_2O_2 induces intracellular acidosis in several cell types, including isolated rat cardiac myocytes[3], renal epithelial cells[4] and human aortic endothelial cells (HAEC)[5]. This is suggested to be due to inhibition of NHE1 activity by depletion of intracellular ATP content[5-9]. In intact cells, ATP is required for optimal function of NHE1[6] and half maximal activation of NHE1 is obtained at about 5 mM ATP[6]. Depletion of ATP induces a marked depression of NHE1 activity in a variety of cell systems[7,9,10]. The mechanism whereby ATP modulates NHE1 remains obscure. Kinetic analysis showed that the predominant effect of ATP depletion is to alter the sensitivity of this exchanger to H^+ activation[6,7].

One of the reasons for H_2O_2 induced-ATP depletion in isolated myocytes is due to its inhibitory effect on glycolysis[11,12]. However, inhibition of glycolysis will also improve the coupling of glucose metabolism, reduce H^+ production and attenuate intracellular acidosis (see 28 for review and Chapter 4 and 5). Therefore, the net effects of H_2O_2 on pH_i may be determined by the relative effects on inhibition of glycolysis and depletion of ATP. Our previous studies have shown that H^+ production generated from uncoupled glucose metabolism, due to the presence of a high level of fatty acid, contributes to ischemia-reperfusion injury by activation of the Na^+/H^+ exchanger (NHE1)[13-15]. Thus, the possibility that H_2O_2, generated during ischemia and reperfusion alters H^+ production and pH_i can not be excluded.

Fatty acids are the major energy source of the heart, and normally provide 60-70% of the hearts energy requirement (see reference 1 for review). During reperfusion, fatty acid oxidation rates quickly recover and provide 90-100% of heart energy requirements[16-18]. Acetyl-CoA carboxylase (ACC) is an important rate-controlling enzyme in the regulation of fatty acid oxidation by generating intracellular malonyl-CoA, a potent inhibitor of mitochondrial fatty acid uptake[16,19]. 5'AMP-activated protein kinase (AMPK) is a major regulator of ACC, phosphorylating the enzyme at unique sites (Ser-77, Ser-1200, and Ser-1215)[16,19] and resulting in enzyme inhibition. Previous studies[19,20] have shown that in reperfused ischemic hearts AMPK activity increases, resulting in a phosphorylation and inhibition of ACC and subsequently reduction of malonyl-CoA production. Since AMPK is a stress-activated protein and can be activated by ATP depletion (see 21 for review), it is reasonable to suggest that H_2O_2

induced-oxidative stress might activate AMPK and up-regulate fatty acid oxidation. Since malonyl-CoA can be decarboxylated by malonyl CoA decarboxylase (MCD)[22,23], it is also possible that H_2O_2 might affect MCD activity and thereby alter fatty acid oxidation. Our previous study has shown that a high rate of fatty acid oxidation inhibits glucose oxidation and delays the recovery of pH_i during reperfusion by increasing H^+ production from uncoupled glucose metabolism (see Chapter 4 for details). Cardiac function and efficiency are subsequently depressed. This is because H^+, generated from uncoupled glucose metabolism, may induce intracellular Na^+ and Ca^{2+} overload via activation of NHE1 and Na^+/Ca^{2+} (NCE). Thus, it is possible that regulation of fatty acid oxidation by H_2O_2 may also affect glucose metabolism and pH_i.

In this chapter, the effects of H_2O_2 on glucose metabolism, fatty acid oxidation and intracellular ATP content were investigated in isolated working rat hearts under normal aerobic conditions. We determined whether alteration of the source of H^+ and ATP content by acute treatment with H_2O_2 can affect pH_i.

2. Methods (for general experimental procedures, please see Chapter 2)

Glycolysis and glucose oxidation were measured simultaneously by perfusing hearts with [5-^{3}H/U-^{14}C]. Fatty acid oxidation rates were measured by perfusing hearts with [1-^{14}C]palmitate. TCA cycle activity was determined by calculating the rate of acetyl-CoA entering the TCA cycle from both glucose oxidation and fatty acid oxidation. Overall rates of H^+ production derived from glucose metabolism were determined by subtracting the rate of glucose oxidation from the rate of glycolysis and multiplying by two. pH_i and high energy phosphate were measured by ^{31}P-NMR. AMPK activity was measured by following the incorporation of ^{32}P into a synthetic peptide and ACC activity was measured using the CO_2 fixation method. Malonyl CoA content was measured by HPLC (see Chapter 2 for details).

3. Experimental Protocol

Isolated working rat hearts were subjected to 70 min of aerobic perfusion. Hearts were randomly assigned to control and H_2O_2 treated-groups. In the control group, working hearts were perfused for a 70-min period under aerobic conditions. In H_2O_2-treated groups, H_2O_2 was added to the perfusate after 30 min aerobic perfusion at a final concentration of either 225 or 450 μM (75 or 150 μM H_2O_2 did not affect cardiac function at any time point and were not included). The hearts were perfused for a further 40-min aerobic period. However, pH_i was monitored for 45 min after the start of 450 μM H_2O_2 treatment. Hearts were perfused with Krebs-Henseleit solution containing 11 mM glucose, 1.2 mM palmitate and 100 μU • ml^{-1} insulin.

4. Results

4.1 Effects of H_2O_2 on cardiac mechanical function

In control hearts, cardiac function was not significantly different throughout the 70 min aerobic perfusion period (Fig. 6-1). However, exposure of hearts to 450 µM H_2O_2 induced a significant decrease in mechanical function. Cardiac work was inhibited by 96±6% after 40-min treatment (Table 6-1, Fig. 6-1). Exposure of hearts to 225 µM H_2O_2 significantly increased coronary flow without affecting other parameters (Table 6-1, Fig. 6-1, and Fig. 6-2). Perfusion with 450 µM H_2O_2 caused a 72±4% decrease in MVO_2 (Table 6-1) and a 82±4% decrease in cardiac efficiency (Fig. 6-1B). In contrast, treatment with 225 µM H_2O_2 did not affect cardiac function and efficiency.

4.2 Effects of H_2O_2 on glycolysis and glucose and palmitate oxidation

Fig. 6-3 shows the amount of substrates metabolized versus time via glycolysis (panel A), glucose oxidation (panel B) and palmitate oxidation (panel C) during 70 min of perfusion. The effects of H_2O_2 on rates of glycolysis and glucose and palmitate oxidation are shown in Table 6-2. The rate of glucose oxidation in control hearts was substantially lower than the rate of glycolysis. This parallels previous observations in isolated working rat hearts perfused with this level of fatty acid[21,22]. Treatment with 450 µM H_2O_2 (but not 225 µM) significantly inhibited glycolysis and palmitate oxidation (Fig 6-3). Of interest, 225 or 450 µM H_2O_2 also increased glucose oxidation rates (Table 6-2).

4.3 Effects of H_2O_2 on pH_i

During 40-min perfusion (30 min to 70 min), treatment with H_2O_2 (225 or 450 μM) did not significantly affect pH_i (Fig 6-4A). However, when cardiac work and coronary flow were completely inhibited after a further 5-min perfusion with 450 μM H_2O_2, pH_i was significantly decreased (Fig 6-4B). In the 450 μM H_2O_2 treatment group, PCr and ATP contents decreased to 51±11% and 46±11% of pretreatment levels, respectively ($p<0.05$), during 40-min perfusion (Fig. 6-5) and a 1.2-fold increase in Pi content was also observed ($p>0.05$). After a further 5-min perfusion with H_2O_2 450 μM, PCr and ATP contents decreased to 10±5% and 21±11% of pretreatment levels, respectively ($p<0.05$). This was associated with a 3-fold increase in Pi content ($p<0.05$). Treatment with 225 μM H_2O_2 did not significantly affect PCr, ATP or Pi contents (Fig. 6-5).

4.4 Effects of H_2O_2 on malonyl-CoA content and MCD activity

Figs. 6-6 and 6-7 show the effects of 40-min perfusion with H_2O_2 (225 or 450 μM) on malonyl-CoA content and MCD activity, respectively. Treatment with 450 μM H_2O_2 (but not 225 μM) significantly increased malonyl-CoA content by 1.8-fold. However, H_2O_2 did not affect MCD activity.

4.5 Effects of H_2O_2 on AMPK and ACC activity

Since AMPK and ACC activity play an important role in the regulation of fatty acid oxidation by regulation of malonyl-CoA content[16], we further investigated whether H_2O_2-mediated inhibition of fatty acid oxidation is related to alteration of these two enzymes. Fig. 6-8 shows that in 450 μM H_2O_2 group, AMPK was increased at the end of 40-min perfusion compared with the control group (630±65 vs 381±77 $\text{pmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$, $p<0.05$). This was paralleled by a decrease in ACC activity (6.7±1.4 vs 11.6±1.1 $\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$, $p<0.05$). Treatment with 225 μM H_2O_2 did not significantly alter AMPK or ACC activity.

4.6 Effects of H_2O_2 on ratio of TCA cycle activity to cardiac work

The effect of H_2O_2 (225 or 450 μM) on the ratios of TCA cycle activity per cardiac work is shown in Table 6-3. Since cardiac work was almost completely inhibited by 40 min treatment with 450 μM H_2O_2, the ratios of TCA acetyl-CoA production per cardiac work (from glucose and palmitate) in 450 μM H_2O_2 treated hearts were dramatically higher than that in control or 225 μM H_2O_2 treated hearts. This resulted in 13-fold increase in the total ratio of TCA acetyl-CoA production per cardiac work in 450 μM H_2O_2 treated group compared with control.

5. Discussion

In the present study, the effects of H_2O_2 on glycolysis, glucose oxidation, fatty acid oxidation, H^+ production and pH_i were investigated in isolated working rat hearts. Although we found that H_2O_2 inhibited cardiac function and depleted intracellular ATP content, in contrast to previous studies, H_2O_2 did not affect pH_i by reducing H^+ production. This was because H_2O_2 inhibited fatty acid oxidation and improved the coupling of glucose metabolism. A decrease in pH_i was only observed after cardiac work and coronary flow had ceased. As a result, we conclude that the adverse effects of H_2O_2 on cardiac function and efficiency are not due to effects on either H^+ production or pH_i.

H_2O_2 is an important mediator in myocardial ischemia/reperfusion injury[1,2]. Previous studies have demonstrated that H_2O_2 is not only produced during reperfusion, but also generated during ischemia, and contributes to ischemic injury[1]. Perfusion of isolated rat hearts with H_2O_2 is a well-established experimental model of cardiac dysfunction caused by reactive oxygen species[24]. In the present study, the effects on H_2O_2 on cardiac function and metabolism in isolated working rat heart perfused with both glucose and fatty acid were investigated. The lower concentration of H_2O_2 (225 μM) caused coronary vasodilatation, which was not accompanied by an increase in cardiac function and MVO_2. In contrast, treatment with 450 μM H_2O_2 almost completely inhibited cardiac function and coronary flow was significantly decreased. A recent study[31] has shown that low concentration of H_2O_2 (25-50 μM) induced vasodilatation and improved the recovery of cardiac function during reperfusion in ischemic rat hearts, while high concentration of H_2O_2 (200 μM) caused an immediate vasodilatation followed by vasoconstriction and inhibited the recovery of cardiac function. The vasodilatation effect observed in above study was proposed to be due to an elevated cGMP level caused by H_2O_2 treatment[30]. Our results suggest that H_2O_2-induced vasodilatation may be dependent on its concentration. The concentrations of H_2O_2 used in this study are actually low. This is because 3% BSA, which was used to bind fatty acids, has a strong scavenging activity against H_2O_2[31,32]. Thus, the actual free concentrations of H_2O_2 in this study may be comparable to those used in previous studies, which did not use fatty acid and BSA in their perfusion[24,32].

Previous studies[3,11,12] have shown that H_2O_2-induced oxidative stress impairs glycolysis and glucose oxidation in rat cardiomyocytes by inhibition of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and PDH. However, the sensitivity of PDH to H_2O_2 overload is markedly less than that of GAPDH[11]. In the present study, our data also showed that the most significant effect of H_2O_2 on myocardial glucose metabolism was an inhibition of glycolysis. This is consistent with previous studies using rat cardiac myocytes or isolated hearts models[3,11,12,26]. Since glucose oxidation is closely associated with cardiac work[27] and H_2O_2 significantly inhibited cardiac mechanical function, it would be expected that glucose oxidation would also decrease. Surprisingly, in contrast to previous studies[11,26], we found that in the isolated working heart model, glucose oxidation was stimulated after acute treatment with H_2O_2. It is widely accepted that down-regulation of fatty acid oxidation can up-regulate glucose oxidation and improve coupling of glucose metabolism in isolated rat hearts (see 16,28 for review). Thus, it is possible that the up-regulated glucose oxidation observed in this study is related to an inhibitory effect of H_2O_2 on fatty acid oxidation.

It is well known that treatment with H_2O_2 induces intracellular ATP depletion. Hu et al[5] have shown that decreasing 40% of intracellular ATP content by H_2O_2 decreases pH_i of HAEC by 0.22 pH unit ($p<0.05$). In the present studies, although PCr, ATP contents and coronary flow decreased to 51±11%, 46±11% and 22±8%, respectively, of pretreatment levels ($p<0.05$), there was no significant change in pH_i. This is not consistent with previous studies demonstrating that H_2O_2 induces intracellular acidosis[3-9]. Besides the different experimental model used in this study (isolated working hearts vs cultured cells), the major reason for above disparity may be related to the presence of palmitate, the major fuel for the heart that was missing in previous studies[3-9]. In the present study, glycolysis was inhibited, while glucose oxidation was increased, resulting in lower H^+ production generated from an improved coupling of glucose metabolism. After 40 min treatment with 450 μM H_2O_2, H^+ production rate was reduced by 62±14% ($p<0.05$). Therefore, treatment with H_2O_2 actually improved the coupling of glycolysis with glucose oxidation, resulting in a low H^+ production rate and this may offset a low pH_i induced by depletion of ATP and reduced coronary flow.

Interestingly, after a further 5-min treatment with 450 μM H_2O_2, cardiac work and coronary flow had completely ceased and pH_i quickly decreased (from 7.16±0.04 to 6.72±0.09, $p<0.05$). This was associated with markedly low levels of PCr and ATP contents (10±5% and 21±11% of pretreatment levels, respectively, $p<0.05$) and a 3-fold increase in Pi content ($p<0.05$). We could not measure glucose or fatty acid metabolism and calculate H^+ production between 40 and 45 min of treatment with 450 μM H_2O_2 since there was no coronary flow. During this period, since the oxidation pathway is completely inhibited, ATP production from glycolysis is the only fuel for the heart and H^+ production generated from hydrolysis of glycolytically derived ATP becomes a major source for intracellular acidosis (see 29 for review). Thus, it is not surprising to demonstrate that pH_i quickly decreased during this period.

Hydroxyl-radical (•OH) or superoxide anion radical ($O_2^{-}\bullet$) generators have been reported to elicit adenylate loss and contractile failure in isolated hearts[25,26]. It is well known that H_2O_2 inhibits cardiac mitochondrial respiration[33,34], inhibits sarcoplasmic reticulum-Ca^{2+} ATPase[35] and alters contractile proteins[36]. All the above mechanisms potentially contribute to H_2O_2-induced cardiac injury. Although a decrease in cardiac function (energy demand) induced by H_2O_2 may decrease fatty acid oxidation, the direct effect of H_2O_2 on fatty acid oxidation can not be ignored. In the present study, we further investigated the mechanisms that H_2O_2 down-regulated fatty acid oxidation. Previous studies have shown that malonyl-CoA, the product of ACC, is a potent inhibitor of mitochondrial fatty acid uptake[16,19,20]. AMPK phosphorylates ACC and decreases its activity, resulting in an increase in fatty acid oxidation[16,19,20]. It was possible that H_2O_2 could have up-regulated fatty acid oxidation by activating AMPK and inhibiting ACC activity. Indeed, AMPK activity was activated and ACC activity was inhibited by 450 µM H_2O_2. However, fatty acid oxidation was inhibited. This may be due to a 1.8-fold increase in malonyl-CoA content. Therefore, the effects of H_2O_2 on AMPK and ACC activities can not explain inhibited fatty acid oxidation in this study. We further investigated whether H_2O_2 could affect the malonyl-CoA degradation pathway. However, MCD activity was not affected. Previous studies demonstrated that malonyl-CoA is widely distributed within different intracellular compartments (including mitochondrial) (see reference 23 for review). It is possible that H_2O_2 generated free radicals can damage intracellular compartments and thus more malonyl-CoA is released. This may overshadow the decreased malonyl-CoA production due to low activity of ACC. Regardless, our results suggest that the inhibitory effect of H_2O_2 on fatty acid oxidation is independent on malonyl-CoA generation or degradation pathways.

Myocardial metabolism is normally finely matched to myocardial performance and changes in contractile function can induce alterations in metabolism. However, Schulz et al[37] found that infusion of peroxynitrite in aerobic perfused working rat hearts (without fatty acid substrate) depressed cardiac efficiency and this was associated with an uncoupling of contractile function from mitochondrial TCA cycle activity. This suggests that mitochondrial ATP production is not efficiently translated into mechanical function. In 450 μM H_2O_2-treated hearts, cardiac function was inhibited 96±6%, while 46±11% of pretreatment level of ATP remained after 40-min perfusion. As a result, there was a marked inefficiency of the heart to utilize the ATP synthesized for mechanical work. This was associated with a 13-fold increase in the total ratio of TCA acetyl-CoA production per cardiac work compared with control. Thus, similar to the effect of peroxynitrite[37], H_2O_2 can also induce an uncoupling of utilization of cardiac energy with contractile function.

One limitation in the present study is that we did not determine whether specific inhibition of NHE1 by cariporide could affect pH_i after depletion of the intracellular ATP. A previous study[5] has shown that inhibition of NHE1 did not further affect H_2O_2-induced intracellular acidosis in HAEC. However, it has been shown that cariporide, a specific inhibitor of NHE1, may have direct antioxidant effects, since the detrimental effects of H_2O_2 on cardiac function can be reversed by treatment with cariporide in isolated rat hearts[38]. A recent study has shown that treatment with cariporide during ischemia does not affect pH_i but attenuates intracellular Ca^{2+} overload[39]. Thus, cariporide may have effects other than inhibition of NHE1.

Summary

Acute treatment with H_2O_2 for 40 min caused a concentration-dependent cardiac depression and decreases in PCr and ATP contents in isolated working rat hearts despite no changes in pH_i. This was associated with an uncoupling of contractile function from mitochondrial TCA cycle activity. H_2O_2 significantly reduced H^+ production via improvement of coupling of glucose metabolism. This may be a major reason that acute treatment with 450 µM H_2O_2 for 40 min did not affect pH_i despite the occurrence of significant reduction in coronary perfusion. However, intracellular acidosis occurred only after cessation of cardiac work and coronary perfusion induced by H_2O_2. This was associated with marked low levels of intracellular PCr and ATP. Fatty acid oxidation was significantly inhibited in H_2O_2 (450 µM)-treated hearts. This may be due to a non-specific release of malonyl-CoA from intracellular compartments. The inhibitory effect of H_2O_2 on fatty acid oxidation may be responsible for the stimulated glucose oxidation in H_2O_2-treated hearts.

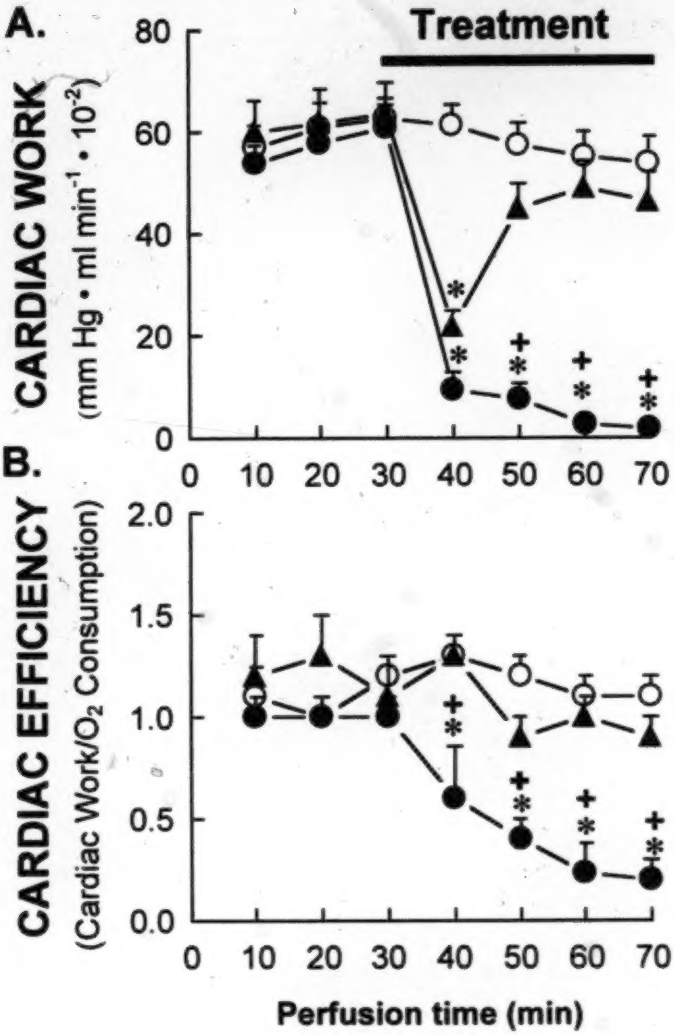

Figure 6-1. Effects of H_2O_2 on cardiac work (A) and cardiac efficiency (B) in isolated working rat hearts.

Values are mean ± SEM. Control, n=8, (O); 225 μM H_2O_2, n=8 (▲); 450 μM H_2O_2, n=8 (●). *, Significantly different from control hearts. $^+$, Significantly different from 225 μM H_2O_2-treated hearts.

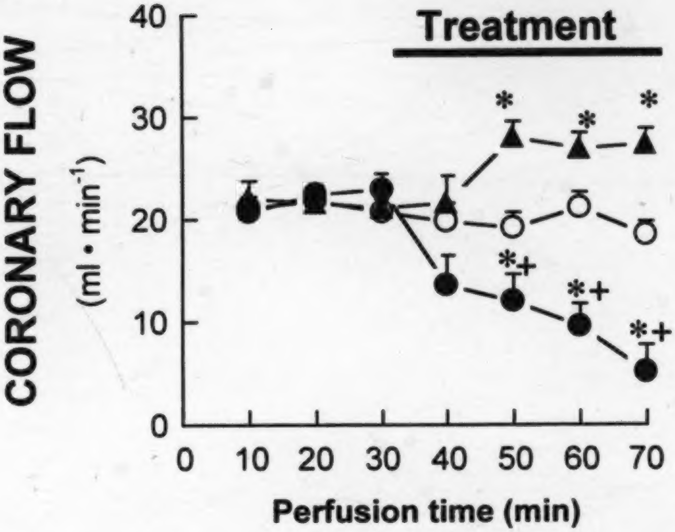

Figure 6-2. The effect of H_2O_2 on coronary flow during aerobic perfusion in isolated working rat hearts.

Values are mean ± SEM. Control, n=8, (O); 225 µM H_2O_2, n=8 (▲);450 µM H_2O_2, n=8,(●). *, Significantly different from control hearts. $^+$, Significantly different from 225 µM H_2O_2 treated hearts.

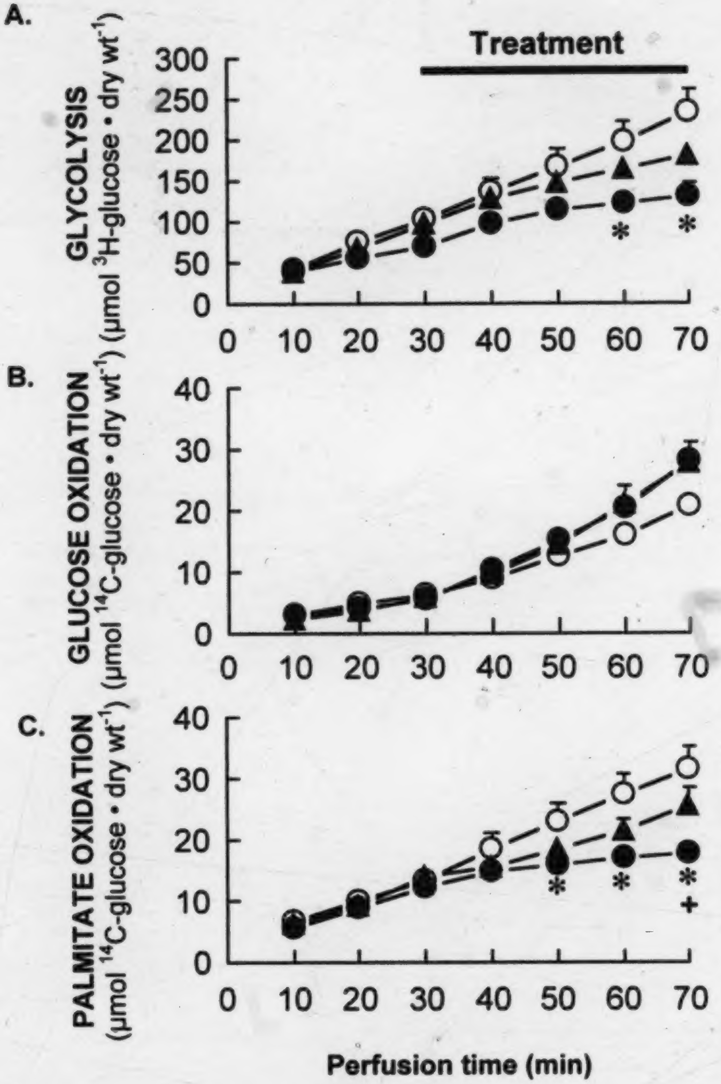

Figure 6-3. Effects of H_2O_2 on the time course of glycolysis (A), glucose oxidation (B), and palmitate oxidation (C) in isolated working rat hearts. Values are mean ± SEM. Control, n=8, (○); 225 µM H_2O_2, n=8 (▲); 450 µM H_2O_2, n=8, (●). *, Significantly different from control hearts. $^+$, Significantly different from 225 µM H_2O_2 -treated hearts.

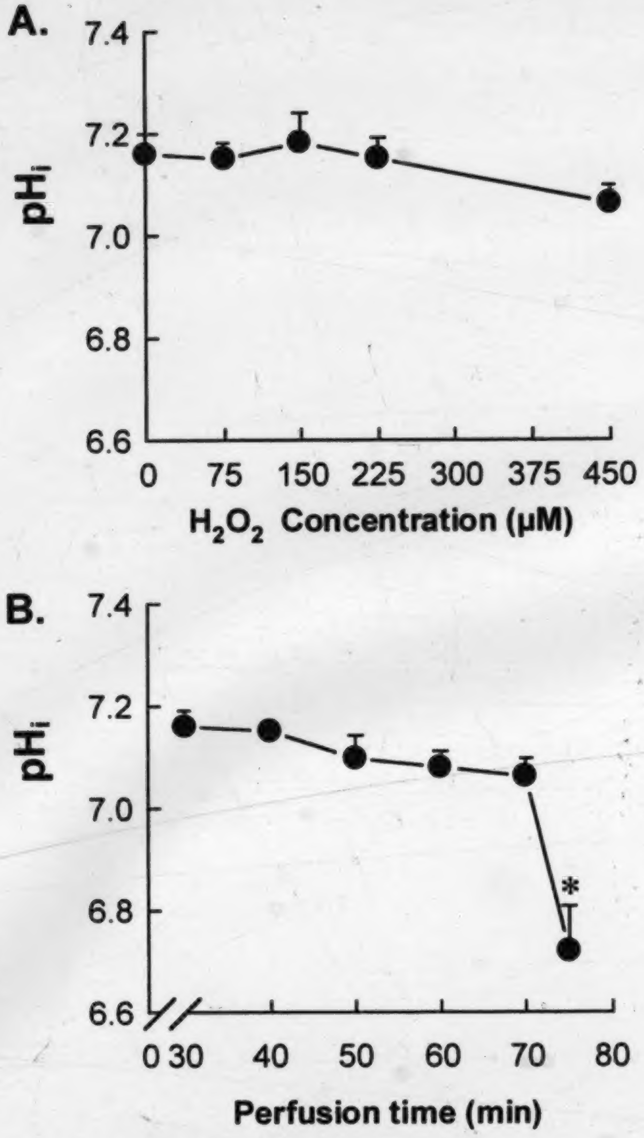

Figure 6-4. Effect of treatment with H_2O_2 on pH_i in isolated working rat hearts. Values are mean ± SEM, n=6. After 30 min aerobic perfusion, (A) hearts were subjected to 40 min treatment with different concentrations of H_2O_2; (B) Hearts were subjected to 45 min treatment with 450 µM H_2O_2. *, Significantly different from pretreatment pH_i value.

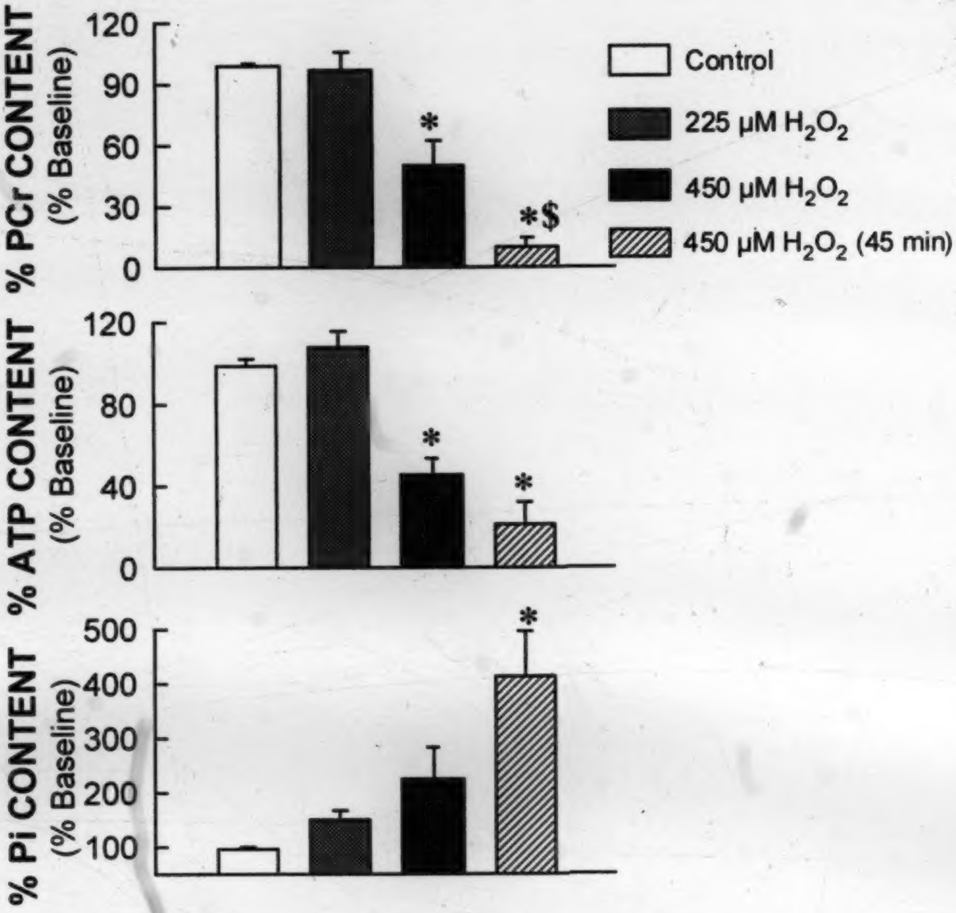

Figure 6-5. Effects of H_2O_2 on PCr, ATP and Pi contents in isolated working rat hearts.

Values are mean ± SEM, n=6. Hearts were subjected to 40 min treatment with H_2O_2 (0, 225 or 450 μM) or 45 min treatment with 450 μM H_2O_2.

*, Significantly different from postreatment values in control and 225 μM H_2O_2 groups. $, Significantly different from postreatment values in 40 min treatment with 450 μM H_2O_2 group.

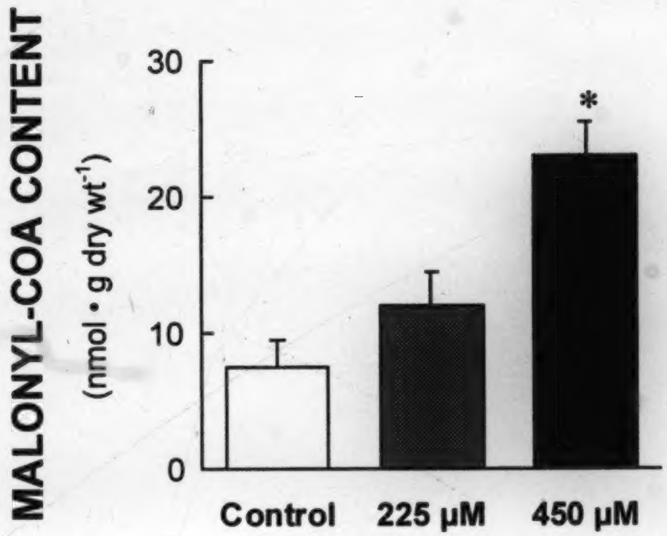

Figure 6-6. Effect of H_2O_2 on malonyl-CoA level in isolated working rat hearts. Values are mean ± SEM, n=8. After 30 min of aerobic perfusion, hearts were subjected to 40 min treatment with 0 (control), 225 or 450 µM H_2O_2, respectively. * , Significantly different from control.

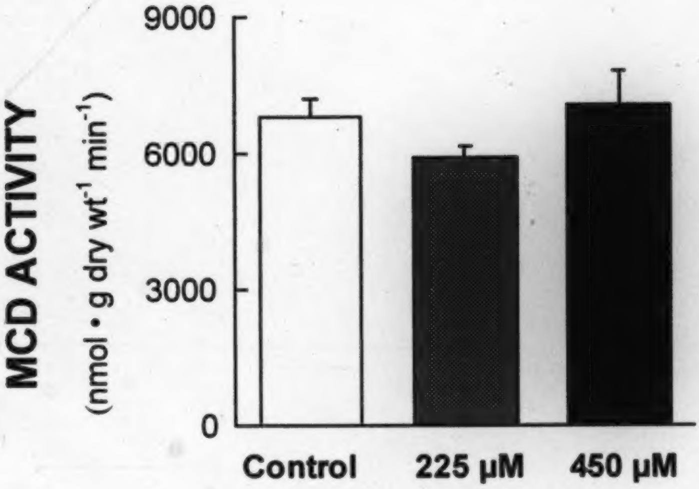

Figure 6-7. Effects of H_2O_2 on MCD activity in isolated working rat hearts. Values are mean ± SEM, n=8. After 30 min of aerobic perfusion, hearts were subjected to 40 min treatment with 0 (control), 225 or 450 μM H_2O_2, respectively.

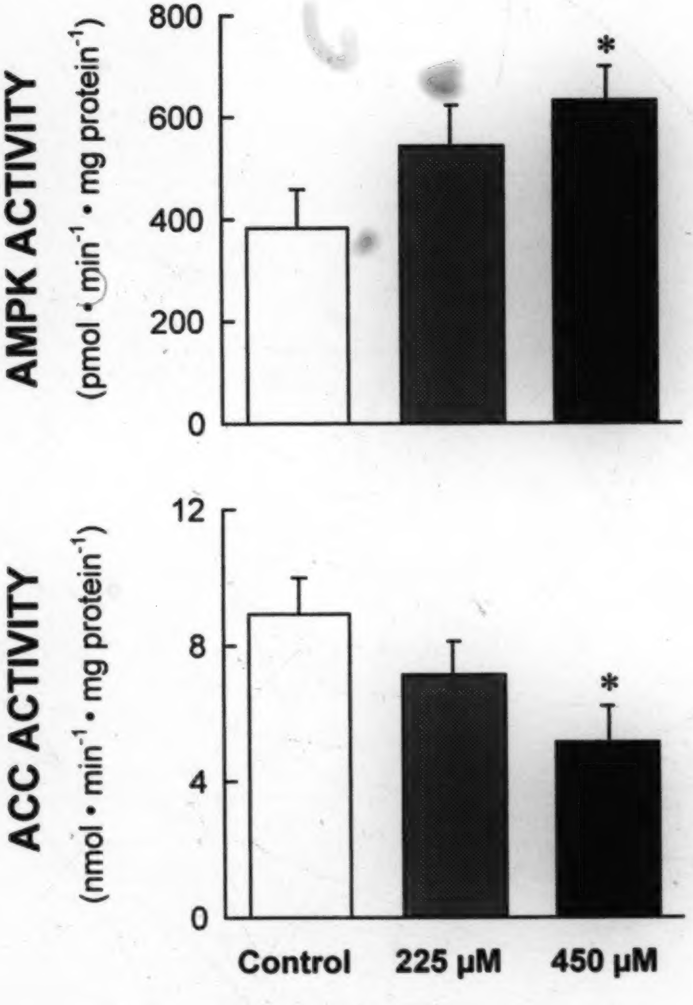

Figure 6-8. Effects of H_2O_2 on AMPK and ACC activities in isolated working rat hearts.

Values are mean ± SEM, n=8. After 30 min aerobic perfusion, hearts were subjected to 40 min treatment with 0 (control), 225 or 450 µM H_2O_2, respectively.

* Significantly different from control hearts.

Table 6-1. Effects of H_2O_2 on the mechanical function of isolated working rat hearts.

Parameter measured		Control (n=8)	225 μM (n=8)	450 μM (n=8)
Heart rate	Pretreatment	249±13	239±12	220±20
(beats • min^{-1})	Postreatment	241±27	211±27	88±11[$+*]
Peak systolic pressure	Pretreatment	130±2	126±4	136±4
(mm Hg)	Postreatment	124±4	108±14	36±14[$+*]
Developed pressure	Pretreatment	74±4	74±4	70±4
(mm Hg)	Postreatment	38±4[+]	34±6[+]	14±6[$+*]
Cardiac output	Pretreatment	48±4	50±5	45±5
(ml • min^{-1})	Postreatment	43±6	42±7	6±3[$+*]
MVO_2	Pretreatment	44±3	50±5	54±4
(μmol • g dry wt^{-1} • min^{-1})	Postreatment	40±3	51±3	15±8[$+*]
Coronary flow	Pretreatment	21±1	21±1	23±1
(ml • min^{-1})	Postreatment	18±1	27±2[*]	5±3[$+*]

Pretreatment values were taken after 30 min perfusion. Postreatment values were taken after 40 min treatment. Values are mean ± SEM; n=8.

[*] Significantly different from postreatment values in control hearts.

[+] Significantly different from pretreatment values in H_2O_2-treated hearts.

[$] Significantly different from postreatment values in H_2O_2 225 μM-treated hearts.

Table 6-2. Effects of H_2O_2 on steady-state rates of glucose oxidation, glycolysis, palmitate oxidation and H$^+$ production in isolated working hearts.

Parameter measured		Control	225 µM	450 µM
(nmol • g dry wt^{-1} • min^{-1})		(n=8)	(n=8)	(n=8)
Glucose oxidation	Pretreatment	156±25	145±35	135±28
	Postreatment	204±47	532±70^{*+}	542±42^{+*}
Glycolysis	Pretreatment	3100±687	2805±467	2655±236
	Postreatment	3213±413	2063±401	1504±220^{*+}
Palmitate oxidation	Pretreatment	456±45	498±56	442±35
	Postreatment	509±69	404±42	242±23^{*+}
H$^+$ production	Pretreatment	5888±612	5320±457	5040±225
	Postreatment	5738±762	3062±443^{*+}	1924±443^{*+}

Pretreatment values were determined between 10 and 30 min. Postreatment values were determined between 40 and 70 min of perfusion. Values are means ± SEM.

* Significantly different from postreatment values in control group.

$^+$ Significantly different from corresponding pretreatment values.

Table 6-3. Effects of H_2O_2 on TCA acetyl-CoA production per cardiac work (CW) from glucose oxidation and palmitate oxidation in isolated working hearts.

TCA acetyl-CoA production per CW from:		Control (n=8)	225 µM (n=8)	450 µM (n=8)
Glucose oxidation	Pretreatment	5±1	5±1	4±1
	Postreatment	$13\pm2^{+}$	$21\pm3^{+}$	$462\pm46^{*+}$
Palmitate oxidation	Pretreatment	45±5	50±7	45±4
	Postreatment	50±8	44±6	$598\pm96^{*+}$
Total	Pretreatment	50	55	49
	Postreatment	81	65	1060

Pretreatment values were determined between 10 and 30 min. Postreatment values were determined between 40 and 70 min of perfusion. Values are means ± SEM.

[*] Significantly different from postreatment values in control group.

[+] Significantly different from pretreatment values.

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Chapter 7

General discussion & conclusions

Despite the fact that H^+ production generated from uncoupled glucose metabolism during reperfusion has been implicated as a contributing factor to the pathophysiology of ischemia-reperfusion injury, a direct determination of whether H^+ production can delay the recovery of pH_i has not previously been made. In the present studies, the impact of both the source and fate of H^+ on the recovery of pH_i, cardiac function and efficiency in postischemic rat hearts was investigated.

Methodology

The continuous contractile activity of heart muscle requires that the myocytes produce large amounts of ATP. The heart meets this ATP demand primarily by metabolizing carbohydrates and fatty acids. Flux through glycolysis, glucose oxidation, lactate oxidation, fatty acid oxidation, TCA cycle activity, and electron transport chain activity are critically dependent on the work performed by the heart. It is therefore critical to use experimental models in which the heart functions at physiologically relevant workloads. For example, oxidative metabolism in isolated cardiomyocytes can be 50 to 100 times lower than rates in intact working hearts due to the quiescent nature of the isolated cardiomyocytes[1,2].

Another important consideration in the measurement of energy metabolism in the heart is the supply of energy substrates to the heart. Due to the complex interrelation among the various metabolic pathways in the heart, altering the supply of a particular energy substrate has considerable impact on flux through the other pathways. For example, removal of fatty acids as a source of energy substrate results in a 5-fold increase in glucose oxidation rates in isolated working rat hearts (see Chapter 4 for details). Although this potential problem is less relevant with *in vivo* studies, it is particularly important in *in vitro* studies. Most *in vitro* heart studies do not provide fatty acids as an energy substrate. As a result, omission of fatty acids artificially increases rates of myocardial glucose metabolism and complicates measurement of energy substrate metabolism and cardiac performance. Thus, in the present studies, measurements of energy metabolism and cardiac function were performed in isolated working rat hearts perfused with both glucose and fatty acid (palmitate).

Use of ^{31}P-NMR allows noninvasive measurement of high energy phosphates (ATP, PCr) and pH_i in the intact heart. However, ^{31}P-NMR does not provide information about which metabolic pathways are responsible for the generation of high energy phosphates. Furthermore, it is well known that levels of ATP are not a good indicator of the recovery of cardiac function in postischemic hearts. In contrast, measurement of energy metabolism using ^{3}H- or ^{14}C-labeled substrates allows direct measurement of flux through the various energy-yielding pathways (carbohydrates and fatty acids, etc.) in the heart. This includes direct measurement of rates of glycolysis, glucose oxidation, lactate oxidation, and fatty acid oxidation. One of the advantages of this approach is that intracellular H^+ production can be calculated from glucose metabolism (see

Chapter 1 for details). Previous studies have indicated that H^+ production in postischemic hearts is detrimental for the recovery of cardiac function in postischemic hearts[3,4]. In the present studies, an isolated working rat heart model for ^{31}P-NMR was developed. Combination of ^{31}P-NMR with ^{3}H- or ^{14}C-labeled radioisotope technique provided us more detail about the relationship between energy substrate metabolism, H^+ production and pH_i in postischemic hearts.

H^+ production, pH_i and regulation of glucose metabolism

In the present studies, T_3 (10 nM) activated PDH, stimulated glucose oxidation and reduced H^+ production. This was associated with significantly improved recovery of cardiac function in postischemic hearts (see Chapter 3 for details). Although this is consistent with previous studies demonstrating that a decreased H^+ production in response to an improvement of the coupling of glucose metabolism is important for the recovery of cardiac function, there was no direct evidence to show that the alteration of calculated H^+ production accounts for the change of pH_i.

Therefore, we investigated whether reduction of H^+ production by stimulating glucose oxidation (omission of fatty acid or stimulation of PDH by DCA) can accelerate pH_i recovery. We found that this is indeed the case. The recovery of pH_i in glucose-perfused or DCA-treated hearts was faster than in glucose+palmitate-perfused hearts during reperfusion (see Chapter 4 for details).

On the other hand, inhibition of NHE1 significantly prolonged intracellular acidosis in postischemic hearts perfused with a high level of fatty acid (Chapter 5). Furthermore, improvement of coupling of glucose metabolism (inhibition of glycolysis and stimulation of glucose oxidation) by H_2O_2 in isolated aerobic working rat hearts also lessened the decrease in pH_i (see Chapter 6 for details). On the basis of the above studies, we propose that an increase in H^+ production generated from uncoupled glucose metabolism, due to the presence of fatty acids, delays the recovery of pH_i in postischemic rat hearts. Reduction of H^+ production by alteration of glucose and fatty acid metabolism can improve the recovery of cardiac function and accelerate the recovery of pH_i. However, the recovery of pH_i *per se* is not the critical factor determining overall recovery of cardiac function. Stimulation of glucose oxidation by DCA did improve the recovery of cardiac function, reduced H^+ production and accelerated the recovery of pH_i during reperfusion. However, inhibition of NHE1 by CAR led to a complete recovery of cardiac function, despite a slower recovery of pH_i during reperfusion. Thus, we conclude it is the clearance of H^+ that is responsible for the ischemia/reperfusion injury during reperfusion (see Chapter 5 for detail). Activation of NHE1 by acidosis generated from uncoupled glucose metabolism may induce intracellular Ca^{2+} overload via NCE during reperfusion.

In the present studies, we have shown that the metabolic generation of H^+ from hydrolysis of glycolytically derived ATP is a major source for intracellular acidosis not only during ischemia (see reference 5 for review), but also during reperfusion. However, the question also arises as to whether H^+ production from hydrolysis of ATP derived from mitochondrial oxidation also contributes to the intracellular acidosis during reperfusion? This is unlikely since that H^+ produced by hydrolysis of ATP, derived from mitochondrial oxidative phosphorylation, are utilized in the re-synthesis of ATP under aerobic condition[5,6]. As a result, there is no net production of H^+ under conditions of oxidative phosphorylation[7].

Inhibition of NHE1 during ischemia and reperfusion

Results from Chapter 5 show that inhibiting NHE1 during ischemia resulted in greater acidification. This suggests that NHE1 is also activated during ischemia. However, some other investigators[7,8] have failed to demonstrate this effect despite the fact that Na^+ and Ca^{2+} loading is attenuated and better recovery of cardiac function occurs. Although a number of studies have shown that inhibition of NHE1 before ischemia can improve the recovery of cardiac function during reperfusion, in the present studies treatment with CAR (5 min before ischemia) led to delayed recovery of mechanical function and efficiency during the first 30 min of reperfusion, despite the fact that cardiac function completely recovered by 50 min of reperfusion (Chapter 5). This may be because pretreatment with CAR significantly slowed the recovery of pH_i, during reperfusion. Furthermore the combination of DCA with CAR prevented the stimulatory effect of DCA on glucose oxidation during the early period of reperfusion. This may be because low pH_i due to pretreatment with CAR may inhibit PDH. In contrast, treatment with DCA alone during reperfusion led to a quick recovery of pH_i, as well as an improvement in glucose oxidation and mechanical function. In future studies it will be necessary to investigate the combined effects of DCA with a lower concentration of CAR administered during reperfusion.

Cause and effect: the relationships between coronary flow, cardiac function and pH_i

It is feasible to argue that low levels of coronary flow observed in the Glucose+Palmitate group (Chapter 4 and 5) during reperfusion may be also responsible for the slow recovery of pH_i and poor recovery of cardiac function and efficiency. However, a report from Schonekess[9] indicates that 1.2 mM palmitate perfusion does not affect coronary flow and cardiac function during 100 min of aerobic perfusion. Furthermore, increasing concentration of palmitate from 0.4 to 1.2 mM does not affect coronary flow and cardiac function during 60[10] or 100[9] min of aerobic perfusion. In the present studies, CVR was not different between Glucose and Glucose+Palmitate groups (see Chapter 4 for details). Thus, it is unlikely that perfusion of 1.2 mM palmitate can directly affect coronary flow in the present studies. Rather, it is the increased H^+ production from uncoupled glucose metabolism, due to the presence of 1.2 mM palmitate, that delays the recovery of pH_i. Clearance of these H^+ via NHE1 leads to a poor recovery of cardiac function and coronary flow.

Limitations of this thesis

Although energy substrate metabolism from exogenous substrates was measured in isolated working rat hearts, the contributions of endogenous stores of triacylglycerol and glycogen were not considered. The mobilization of endogenous triacylglycerol stores occurs in rat hearts perfused in the absence of fatty acids[11]. However, mobilization decreases when fatty acid concentration in the perfusate is increased[11]. This reduces the likely contribution of triacylglycerol stores to energy production in these studies (except in glucose-perfused group). Although glycogen mobilization can also contribute to energy production and H^+ production in the heart, it is unlikely to play a significant role in the present studies because there was no difference in pH_i between Glucose and Glucose+Palmitate groups, suggesting that glycogen mobilization could not account for our results.

Summary and conclusion

On the basis of the results presented in this thesis, it is concluded that H^+ production generated from uncoupled glucose metabolism due to the presence of a high level of fatty acid delays the recovery of pH_i in the postischemic heart. Clearance of H^+ by activation of NHE1 leads to a poor recovery of cardiac mechanical function and efficiency during reperfusion. This is because: 1) in the presence of high level of fatty acid, glucose oxidation is inhibited, resulting in an increased H^+ production generated from uncoupled glucose metabolism. This is associated with a slower recovery of pH_i and a poor recovery of cardiac function and efficiency during reperfusion; 2) reduction of H^+ production, by either activating glucose oxidation or inhibiting glycolysis, accelerates the recovery of pH_i; 3) either inhibiting NHE1 or decreasing H^+ production by activating glucose oxidation improves the recovery of cardiac work and efficiency. Thus, decreased H^+ production by optimizing energy substrate preference by the heart during reperfusion is a potentially exciting new approach to treating ischemic heart disease.

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Chapter 8

Future directions

From the data revealed in my thesis research, there are several questions that remain to be answered regarding the regulation of energy metabolism and intracellular ionic homeostasis in postischemic hearts.

Measurement of intracellular Na^+ and Ca^{2+}

From the present studies the question arises as to whether the decrease in H^+ production, by improving the coupling of glucose metabolism, can attenuate the increase in intracellular Na^+ and Ca^{2+} concentrations? It is well known that inhibition of NHE1 during reperfusion attenuates intracellular Na^+ and Ca^{2+} overload in postischemic rat hearts. As discussed in Chapter 5, reduction in H^+ production may result in less activation of NHE1. It is therefore reasonable to assume that similar to the effects of inhibition of NHE1, a reduction in H^+ production by improvement of the coupling of glucose metabolism may also attenuate intracellular Na^+ and subsequently Ca^{2+} accumulation during reperfusion. Intracellular Na^+ and Ca^{2+} can be measured by ^{23}Na-NMR and ^{19}F-NMR, respectively.

Down-regulation of fatty acid oxidation and the recovery of pH_i and cardiac function

It is well known that down-regulation of cardiac fatty acid oxidation leads to up-regulation of glucose oxidation (see Chapter 1 for details). Thus, it is feasible to assume that inhibition of fatty acid oxidation can improve the coupling of glucose metabolism, reduce H^+ production and accelerate pH_i recovery and improve the recovery of cardiac function in postischemic hearts. Future studies will be required to address the issue that whether inhibition of fatty acid oxidation can accelerate the recovery of pH_i, attenuate Na^+ and Ca^{2+} overload and improve the recovery of cardiac function during reperfusion.

Roles of other H^+ extrusion pathways (MCT and vacuolar-H^+ ATPase) on cardiac function and pH_i

It is well documented that MCT and vacuolar-H^+ ATPase play an important role in maintaining pH_i within the normal range (see Chapter 1 for details). Unlike NHE1, these two H^+ extrusion pathways clear H^+ from the cell without causing Na^+ accumulation. Since there is a close relationship between cardiac energy metabolism and MCT (see Chapter 1 for details),. it would be interesting to investigate the impact of MCT (inhibition or activation) on the recovery of pH_i, cardiac energy metabolism and cardiac function in postischemic hearts.

Recently, the Gottlieb's group[1] demonstrated vacuolar-H^+ ATPase plays an important accessory role in cardioprotection by reducing acidosis and NHE1-induced Ca^{2+} overload. It is estimated that inhibition of vacuolar-H^+ ATPase can delay the recovery of pH_i and enhance activation of NHE1, resulting in poor recovery of cardiac function in postischemic working hearts. Future studies should address the role of vacuolar-H^+ ATPase on the recovery of pH_i and cardiac function in our model. It would also be interesting to investigate the relationships between cardiac energy metabolism and vacuolar-H^+ ATPase, particularly as ATP content plays an important role in the regulation of its activity[2,3].

Roles of free radicals in regulation of energy metabolism and pH_i

Although the effect of H_2O_2-induced acidosis is well documented, it is not clear whether peroxynitrite, which is one of the major free radicals generated during reperfusion, also causes intracellular acidosis. It has been shown that peroxynitrite stimulates glycolysis during reperfusion[4]. It is therefore reasonable to assume that peroxynitrite can induce intracellular acidosis and subsequently stimulate NHE1. This may contribute to its detrimental effects on the recovery of cardiac function and efficiency. Since free radicals play important roles in ischemia and reperfusion injury and it appears that H_2O_2 and peroxynitrite may affect cardiac pH_i by different mechanisms, it would be interesting to investigate how they affect cardiac energy metabolism and recovery of pH_i during reperfusion.

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